EVALUATION OF TWO ^{15}N METHODS FOR MEASURING NITROGEN FIXATION BY LEGUMES IN ESTABLISHED PASTURES

by

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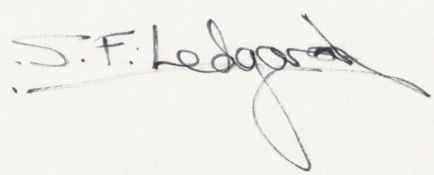
STATEMENT

The work presented in this thesis is my own. Specific contributions by others have been referred to in the text and acknowledgements.



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The following papers, containing results from this thesis, have been accepted for publication:

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ERRATA

Page	Line	Amendment
(iii)	20	Replace actylene by acetylene.
(ix)	1	Replace absortpion by absorption.
18	23	Replace The found by They found.
64	11	Replace aperature by aperture.
81	6	Replace isotopic fraction by isotopic fractionation.
116	9	Replace sulphydral by sulphydryl.
133	17	Replace Virtenan by Virtanen.
134	24	Replace (Table 3.1) by (Table 3.2)
163	8	Replace N = ref. N by N = 2 x ref. N.
201	24	Replace P_o) by P_o (remove bracket).
201	25	Replace On plot by One plot.

CONTENTS

TERMINOLOGY AND SYMBOLS

Different terms have been used in the discussion of data on the stable isotope, ^{15}N. The term 'natural ^{15}N abundance' refers to ^{15}N concentrations of nitrogen in unamended soil or plant material and is generally expressed as $\delta^{15}N$. The term '^{15}N enrichment' describes the ^{15}N concentration of nitrogen in soil or plant material that has been labelled by adding ^{15}N-enriched combined nitrogen and specific values are given as atoms % ^{15}N. The general terms '^{15}N concentration' and 'isotopic composition' are used synonymously to describe either labelled or unlabelled material.

The term 'nitrogen yield' is used in explicit descriptions (e.g. mg N pot^{-1}, kg N ha^{-1}) and is used synonymously with the 'amount of nitrogen' and 'nitrogen accumulation'.

Throughout this thesis, statistical comparisons involve the following terms and symbols:

variance	is used in general descriptions of error;
S.E.	is the standard error; and
S.E.D.	is the standard error of the difference between two or more means.

Symbols which are used frequently are as follows;

B	the ^{15}N concentration of legume nitrogen derived entirely from atmospheric N_2
$\underline{P}$	the proportion (%) of legume nitrogen fixed from atmospheric N_2
$\underline{R}$	the ratio of nitrogen assimilated by plants from added combined nitrogen and from indigenous soil nitrogen.

SUMMARY

Increasingly the natural ^{15}N abundance and ^{15}N isotope dilution methods are being applied to the measurement of N_2 fixation by legumes in the field, but the accuracy of these methods is uncertain. Therefore, the study reported in this thesis was initiated to examine the validity of some of the assumptions upon which the methods are based, with particular reference to mixed legume/grass pastures.

The natural abundance method depends on the ^{15}N concentration of soil N being significantly different from that of atmospheric N_2. In a range of pasture soils (0-50 mm depth) the $\delta^{15}N$ of total N varied from 2.55 to 6.79‰ (with respect to atmospheric N_2). Within soil profiles, the $\delta^{15}N$ of total N increased with soil depth, but there was no significant change with depth in the $\delta^{15}N$ of N assimilated by plants.

Isotopic fractionation occurred during N_2 fixation by three legumes and it was necessary to adjust for this, in the calculation of the proportion (P) of legume N fixed from atmospheric N_2, using the natural ^{15}N abundance method.

Field studies with four legume/grass associations were used to examine further features of the natural abundance and isotope dilution methods that could affect the estimate of P. Errors associated with P were slightly higher when the natural abundance method was used. The ^{15}N isotope dilution method produced:

1) different estimates of P when the $^{15}NO_3^-$ was washed into the soil with different amounts of water, and
2) different estimates of P using different reference plants, even when sampled from within the same plot.

The second factor has the largest effect on the estimates of P using ^{15}N isotope dilution.

In order to evaluate objectively the suitability of reference plants in ^{15}N isotope dilution studies, a technique was developed so that the ratio (R) of N assimilated from the added ^{15}N-labelled N and indigenous soil N could be measured separately for the legume and reference plant. This technique was used in a soil profile experiment in which negative estimates of P were obtained by the ^{15}N isotope dilution method for a clover/phalaris association during the first 16 days after addition of $^{15}NO_3^-$. The latter result was associated with R_{clover} exceeding $R_{phalaris}$; in turn this was due to a declining enrichment of ^{15}N in the plant-available soil N and a greater initial uptake of N by clover. When ^{15}N isotope dilution was used on a clover/ryegrass association, there was no significant difference in R between these two plants. However, estimates of P using natural abundance for clover grown with ryegrass were higher than when clover was grown with phalaris; this was associated with lower levels of inorganic soil N in the clover/ryegrass association. Thus, there may be errors in the estimate of P using ^{15}N isotope dilution caused by mismatching of temporal patterns of N assimilation by the legume and reference plant. Also, the reference plant may influence P in mixed legume/grass associations because different grasses may differentially lower soil N levels.

These studies indicate that care should be taken in using the ^{15}N isotope dilution method, particularly in relation to choosing a suitable reference plant, if reliable estimates of P are to be obtained. In this respect, the natural ^{15}N abundance method was less susceptible to errors in estimating P. However, it is more prone to

problems associated with isotopic fractionation during N_2 fixation and sample preparation; these should be examined critically when the natural ^{15}N abundance method is used.

CHAPTER 1

INTRODUCTION

Nitrogen is one of the most widely distributed elements in nature. Most of it occurs in the dinitrogen (N_2) form in rocks and sediments in the earth's crust and the amount present is about 50 times that found in the atmosphere (Burns and Hardy 1975). However, the soil layer contains only a minute fraction of the lithosphere nitrogen (N) and only a very small proportion of the soil N is directly available to plants (Stevenson 1982).

Plant-available soil N occurs predominantly in the form of nitrate, nitrite and ammonium and the amount of each of these forms can vary considerably over short periods due to biological mineralization, immobilization and assimilation processes (Young and Aldag 1982), but in general it is very small. In addition, large losses of plant-available soil N can occur by biological denitrification and by the physical processes of erosion, leaching and volatilization (Harmsen and van Schreven 1955; Ball 1979; Steele and Shannon 1982). The input of N in precipitation is normally in the range of 0.8-22.0 kg N $ha^{-1}yr^{-1}$ and is generally too small to be of importance in crop and pasture production (Stevenson 1982). Thus, most soils are deficient in N for plant growth (Subba Rao 1977). Nitrogen must be supplied in fertilizers or by growing legumes to fix atmospheric N_2 if high yielding crops and pastures are to be grown.

World use of fertilizer N is huge, being over 60 million tonnes during 1980/81 (F.A.O. 1981). In the United States of America the manufacture of N fertilizer uses more energy than any other single facet of agricultural production (Keeney 1982). There is a vast

supply of 'free' atmospheric N_2 but at ordinary temperatures and pressures it can only be utilized by N_2-fixing microorganisms. The symbiotic association formed between N_2-fixing microorganisms and crop or pasture legumes results in the conversion of atmospheric N_2 to plant N, which eventually becomes incorporated into the soil organic matter through plant death and decay, or via the grazing animal in the form of dung and urine (Simpson 1976; Dobereiner and Campello 1977; Mulder et al. 1977; Franco 1978; Hamdi 1982). However, additions of biologically-fixed N from crop legumes are insufficient to provide enough protein N to meet world demands (Subba Rao 1977). Similarly, N deficiencies still occur in legume-grass pastures (Mulder et al. 1977; Ball et al, 1978; Steele and Shannon 1982). Furthermore, Ball (1979) found that intensively grazed pasture systems with active N_2-fixing clovers had a net loss in total soil N of 70-120 kg N $ha^{-1}yr^{-1}$. Thus, Postgate (1980) noted that it is "probably obligatory during the next century" to increase the exploitation of biological N_2 fixation in agriculture. This comment was based on the increasing concern about the escalating costs, in energy and environmental pollution, incurred in the production and use of nitrogenous fertilizers.

Increased N_2 fixation by legume-rhizobium associations can occur with more efficient host-strain combinations (Mulder et al. 1977) and by overcoming limitations to legume growth and N_2 fixation from nutrient deficiencies in the soil (Gibson 1977). However, before it is possible to increase N_2 fixation, it is essential to be able to measure N_2 fixation accurately in the field. Only then will it be possible to fully understand the influences of environmental and management practices on N_2 fixation.

Numerous methods have been used to estimate N_2 fixation by legumes in the field, but there is a problem of distinguishing between biologically fixed N and that absorbed from the soil. The most promising methods involve the use of the stable isotope of N, ^{15}N (Jansson 1971; Hauck and Bremner 1976). In particular, two methods utilising either natural or artificial enrichment of ^{15}N in soils have been used but the accuracy and reliability of these methods have not been adequately evaluated.

Experiments were therefore undertaken between 1981 and 1984 to examine the validity of the basic assumptions which are implicit and explicit in the ^{15}N methods for measurement of N_2 fixation. Detailed studies were conducted under glasshouse conditions and more general studies on the application of the ^{15}N methods were made in the field on pastures containing nodulated subterranean clover (Trifolium subterraneum L.) or lucerne (Medicago sativa L.).

CHAPTER 2

REVIEW OF LITERATURE : METHODS FOR ESTIMATING N_2 FIXATION BY LEGUMES IN THE FIELD

2.1 Introduction

The estimation of N_2 fixation has often been made by growing legumes in an artificial N-free medium (Dart and Day 1971; Lie 1971; Nutman et al. 1971; Oghoghorie and Pate 1971). Under these conditions, determination of total plant N is all that is required for an assessment of N_2 fixed. However, in the field, legumes have access to inorganic soil N in addition to atmospheric N_2 and there is an inverse relationship between the amount of N fixed and the amount of soil N assimilated (McAuliffe et al. 1958; Allos and Bartholomew 1959). The amount of soil N available for uptake by the legume varies with time due to the cycling of N between the soil pools which vary in their availability to plants (Jansson 1971). Within legume-grass pastures, the associated grasses compete with the legume for the available soil N and consequently less soil N is assimilated by the legume. This generally results in a high proportion of legume N being fixed from atmospheric N_2 (Walker et al. 1956). The amount of growth and N_2 fixation by legumes is also affected by competition from grasses for light, moisture and soil nutrients other than N (Donald 1963; Haynes 1980). Thus, there are numerous factors in the field ecosystem which interact to determine the level of N_2 fixation by legumes, making the estimation of N_2 fixation difficult.

In the past, N balance and acetylene reduction have been the main methods used to estimate N_2 fixation by legumes in the field but during the past seven years there has been an increase in the use of ^{15}N methods. Estimates of N_2 fixation by pasture legumes in the field using these methods are given in Table 2.1. A large variation in estimates is apparent, presumably due to differences in legume species, environment, soil, management and possibly method of measurement.

This review discusses the various indirect and direct methods which have been used for estimating N_2 fixation by legumes in the field, with special emphasis on the estimation of N_2 fixation by pasture legumes. In particular the ^{15}N methods are described and any assumptions associated with their use are discussed in detail.

2.2 Indirect Methods

2.2.1 Ureide method

The first stable free product of N_2 fixation in all legume nodules is ammonia and this is assimilated to glutamine and glutamate (reviewed by Bergersen 1982). These products undergo further assimilation in the nodule before translocation to other plant parts. There are two broad groups of plants which differ in their translocation products, viz. those in which N is translocated predominantly as amides, such as asparagine, and those in which the major translocation product is ureide-N (allantoin + allantoic acid). In the latter group, the production of ureides has been found to be specific to the nodule tissue (Matsumoto et al. 1977). Therefore, the level of ureide-N being translocated decreases as the proportion of legume N obtained from soil inorganic N increases and it has been

Table 2.1. Estimates of N_2 fixation by pasture legumes in the field.

	Measurement technique	Legume	kg N fixed ha^{-1} yr^{-1} Mean	Range
U.S.A.				
Williams et al. 1977	A-value	sub. clover[5]	93	
West and Wedin 1981	^{15}N-ID[1]	lucerne[6]	70	33-104
Heichel et al. 1981b	^{15}N-ID	lucerne	165	159-171
	^{15}N-ID	red clover[7]	109	98-119
Canada				
Rice 1980	A.R.[2]	alsike clover[8]	61	8-143
	A.R.	red clover	35	6- 77
United Kingdom				
Cowling 1961	LN[3] + TN[4]	white clover[9]	185	74-280
Halliday and Pate 1976	A.R.	white clover	268	246-311
Nutman 1976	N difference	lucerne	225	90-342
New Zealand				
Sears et al. 1965	LN + Δ soil N	white clover	477	332-609
Brock 1973	LN + Δ soil N	white clover	486	402-570
	LN + Δ soil N	suckling clover[10]	230	196-264
	LN + Δ soil N	Lotus pedunculatus	502	412-591
Edmeades and Goh 1978	^{15}N-ID	white clover	92	45-142
Hoglund et al. 1979	AR	white clover	185	85-342
Australia				
Lapins and Watson 1970	LN	sub. clover	161	
Simpson et al. 1974	Δ soil N	sub. clover	51	29- 68
Simpson 1976	Δ soil N	sub. clover	159	
	Δ soil N	white clover	483	
	Δ soil N	lucerne	322	
Johansen and Kerridge 1979	LN + TN	tropical species[11]	102	51-137

[1] Isotope dilution; [2]Acetylene reduction; [3]Total legume N; [4]Transferred N_2 from legume to grass; [5]Trifolium subterraneum L.; [6]Medicago sativa L.; [7]T. pratense L.; [8]T. hybridum L.; [9]T. repens L.; [10]T. dubium Sibth.; [11]Desmodium intortum, Glycine wightii, Lotononis barnseii and Macroptilium atropurpureum.

suggested that the relative ureide content [ureide-N/(ureide-N + nitrate-N)] of the xylem sap may be used to indicate the proportion of N_2 fixed by this particular group of legumes (McClure *et al*. 1980; Herridge 1982b).

This method is limited to those legumes that produce ureides and they are largely the crop legumes e.g. soybeans (*Glycine max* [L.] Merr.) and cowpeas (*Vigna unguiculata* [L.] Walp.)(McNeil 1982). In the main pasture legumes, e.g. clover species and lucerne, amides are the predominant products translocated from root nodules (Atkins 1982) and therefore this method cannot be used for estimating N_2 fixation by these plant species.

This is an indirect method for estimating N_2 fixation because it is necessary to establish a relationship between the relative ureide content of the legume and the proportion of legume N obtained from atmospheric N_2. Herridge (1982a) obtained a high correlation ($R^2 \simeq 0.9$) between the relative level of ureides and the proportion of N fixed from the atmosphere, for soybeans grown in sand culture. However, he found that this relationship changed with plant age, making it necessary to establish standard curves with time or to take regular samples throughout the growth period of the plant.

McClure *et al*. (1980) extracted ureides from the xylem sap of soybeans by a root-bleeding technique. However, this sampling procedure is not practical in field studies because of the difficulty in collecting sap from unwatered plants (Herridge 1982a). Instead, Herridge (1982a,b) obtained ureide samples from plant leaf extracts. In both extraction techniques it must be assumed that the samples are representative of the whole plant.

Although the ureide method is useful for qualitative purposes, LaRue and Patterson (1981) considered that this method is unlikely to be of use for quantitative estimation of N_2 fixation by legumes. Nevertheless, the ureide method has recently been used with some success for estimation of N_2 fixation by irrigated soybeans in Australia (Herridge et al. 1984).

2.2.2 Acetylene reduction method

Early work revealed that the nitrogenase enzyme involved in the reduction of atmospheric N_2 to NH_3 will also catalyse the reduction of acetylene (C_2H_2) to ethylene (C_2H_4) (Koch and Evans 1966; Hardy et al. 1968). This led to the acetylene reduction method, which generally involves enclosure of the legume in a sealed container and the addition of C_2H_2 to a concentration of 0.05 to 0.10 bars. At this level the reduction of N_2 by the nitrogenase enzyme is completely inhibited and instead the enzyme converts C_2H_2 to C_2H_4 (Hardy and Holsten 1977). After a fixed period of incubation, gas samples are collected and analysed for C_2H_4 by gas chromatography (Hardy et al. 1968; Havelka et al. 1982). There has been widespread use of the acetylene reduction method during the past 20 years due to its rapidity, simplicity, high sensitivity and low equipment and resource costs (Turner and Gibson 1980).

The acetylene reduction method is an indirect method of measuring N_2 fixation and a conversion factor must be used to relate the moles of C_2H_2 reduced to the moles of N_2 fixed. The theoretical C_2H_2/N_2 conversion factor is 3:1 because six electrons are required to reduce N_2 to NH_3 whereas only two electrons are used for the reduction of C_2H_2 to C_2H_4. However, measurements of the conversion ratio have ranged from 1.5:1 to 25:1 (Hardy et al. 1973).

The nitrogenase enzyme is also capable of reducing H^+ to H_2, and variability in the production of H_2 during N_2 fixation is considered to be a major factor affecting the C_2H_2/N_2 conversion ratio (Havelka et al. 1982). Hydrogen production is inhibited by C_2H_2 and therefore during an acetylene reduction assay the electrons normally involved in H_2 production are redirected to reduce C_2H_2 to C_2H_4 (Schubert and Evans 1976). Thus it has been strongly recommended that studies with C_2H_2 reduction also involve studies to determine the true C_2H_2/N_2 conversion ratio (Hardy and Holsten 1977).

A fundamental assumption of this method is that the rate of nitrogenase activity is not affected by the change in substrate from N_2 to C_2H_2 or the change in end-product from NH_3 to C_2H_4. Minchin et al. (1983) found this assumption to be false for a range of legume species and assay conditions. They measured a large decline in nitrogenase activity within a few minutes of exposure to C_2H_2 and associated this with a concurrent decline in root respiration.

The application of the acetylene reduction method in the field has commonly involved assaying samples of nodules, roots or whole plants in gas-tight vessels (Havelka et al. 1982), although some continuous flow in situ techniques have been used (Hardy et al. 1977). In white clover pastures, Sinclair et al. (1976) and Goh et al. (1978) found that the best sampling method was to collect many small soil cores for each assay vessel but they still found that variability was large due to irregular spatial distribution of N_2 fixation in the field.

In applying the acetylene reduction method in the field it is assumed that all of the C_2H_4 has been derived from the reduction of

C_2H_2 by N_2-fixing bacteria. Many soil systems produce (Smith 1976) and oxidize (Cornforth 1975) C_2H_4 and this should be allowed for in the calculation of the amount of N fixed. A control treatment without C_2H_2 is commonly used to assess the net endogenous production of C_2H_4. However, the addition of C_2H_2 blocks the oxidation of C_2H_4 causing an accumulation of C_2H_4 from endogenous sources as well as from C_2H_2 reduction and therefore N_2 fixation is overestimated (Witty 1979). Since endogenous production of C_2H_4 in soil is small, this will only be important at low rates of nitrogenase activity.

The period of incubation of samples with C_2H_2 is important in obtaining a valid estimate of N_2 fixation. This must be sufficient to allow the C_2H_2 to equilibrate in the assay vessel but not long enough to depress the rate of C_2H_4 production (Goh et al. 1978). In the field, incubation periods of 30 minutes to two hours have commonly been used (Halliday and Pate 1976; Sinclair et al. 1976). However, the rate of C_2H_4 production is subject to marked diurnal, day-to-day and seasonal variations (Halliday and Pate 1976; Sinclair et al. 1976; Eckart and Raguse 1980; Rice 1980). This makes it necessary to integrate numerous short-term measurements and for this reason Halliday and Pate (1976) and Goh et al. (1978) concluded that the C_2H_2 reduction method is unsuitable for long-term quantitative estimation of N_2 fixation by pasture legumes in the field. However, it can be a useful method for making short term comparisons between treatments (e.g. Ledgard and Saunders 1982).

2.2.3 Response by non-legumes

Fixation of N_2 has also been estimated by two indirect methods involving measurement of the response by non-legumes to the presence of legumes. In one method, the increase in N yield of the

non-legume is measured and in the other, this response is compared to that of the non-legume grown with a range of rates of N fertilizer. In these methods the non-legume may be grown with, or subsequent to the growth of, a legume.

These methods obviously do not measure N_2 fixation directly but provide a measure of the effect of legume growth on the concurrent or subsequent N accumulation by a non-legume. Thus, they only estimate fixed N_2 which has been transferred to the non-legume and not that which has been incorporated into the slowly-available soil organic matter or lost from the plant/soil system by leaching, denitrification, volatilization and erosion.

2.2.3.1 Increase in nitrogen yield by non-legumes

With crop legumes, N_2 fixation can be estimated by measuring the amount of N assimilated by a non-legume following a period of growth by a legume, and comparing this with the amount of N assimilated by the non-legume following a control. This control is either a non-legume or bare soil (fallow) and is managed separately and concurrently to the legume. With this method, Askin et al. (1982) measured 73% higher wheat (Triticum aestivum L.) yields when grown subsequent to several grain legumes than when grown after a control crop of barley (Hordeum vulgare L.). A major problem with this method utilising a crop grown subsequent to the legume is that the soil N status should remain unchanged during the growth of the control and this is virtually impossible to achieve. Accurate estimation of N_2 fixation by this method also depends on all of the fixed N_2 being assimilated by the non-legume. However, Ladd et al. (1983) found that less than 30% of the N in Medicago littoralis material incorporated into the soil was recovered by two successive crops of wheat.

With pasture legumes, N_2 fixation can also be estimated by growing the non-legume in association with the legume, thereby estimating a more direct effect of N_2 fixation by the legume and not a subsequent effect of a period of growth by the legume. In using this method, Herriot and Wells (1960) found that the average rate of N accumulation by ryegrass (Lolium perenne L.) and cocksfoot (Dactylis glomerata L.) was 88 and 71 kg N ha^{-1} yr^{-1} higher when grown with white clover than when they were grown alone. Similarly, Simpson (1976) found that the transfer of N from white clover to cocksfoot was 88 kg N ha^{-1} over a three year period, but this was small compared with the increase in soil N (0-10 cm) due to white clover of 483 kg N ha^{-1}. Haystead and Marriott (1978) also found that the estimate of N_2 fixation by measurement of the direct transfer of N from white clover to ryegrass was very small (< 6% of the total) compared with that using total N accumulation (section 2.3.1) or ^{15}N isotope dilution (section 2.4.3) techniques.

2.2.3.2 Nitrogen fertilizer equivalence

With this method the amount of N fixed by the legume is estimated indirectly by assessing the amount of fertilizer N required to produce the same growth response by a non-legume to the presence of a legume. In the case of crop legumes, this usually involves the growth of a non-legume subsequent to a period of growth by the legume. For example, Kroontje and Kehr (1956) estimated that the amount of N fixed by a vetch (Vicia villosa L.) crop was 107 kg N ha^{-1} by comparing the response of barley to the vetch crop and to additions of ammonium nitrate.

With pasture legumes, N_2 fixation is estimated from the response to N fertilizer by grass grown in a pure stand in comparison to the

yield of grass grown with a legume. In this way, Wagner (1954) found that the amount of N transferred from white clover to cocksfoot over a two year period was equivalent to 142 kg fertilizer N ha^{-1} yr^{-1}. Similarly, Cowling (1961) found that the effect of white clover on cocksfoot growth was equivalent to that where 83 kg N ha^{-1} was applied as nitrochalk. This is higher than his estimate of N_2 fixation of 68 kg N ha^{-1} based on the increase in N yield by cocksfoot grown with white clover than for cocksfoot grown alone (section 2.2.3.1).

When this method is used it is assumed that the response by non-legumes to fertilizer N can be compared with that from N released from decomposing legume tissue. This is unlikely because losses of fertilizer N from the soil will probably exceed those from legume N, due to all of the fertilizer N being in the soluble form and being added in one large application. In contrast, with legume N there is a slow continuous release of plant-available N with time due to microbial decomposition and this is more likely to maintain the rate of N assimilated by the non-legume than with fertilizer N. The greater loss of fertilizer N than legume N would cause an overestimation of N_2 fixation.

2.3 Direct methods

2.3.1 Total nitrogen accumulation

The simplest, but not the most accurate method of estimating N_2 fixation is to measure the total N accumulated by the legume during its growing period. When this method is used it is assumed that the legume obtains all of its N by symbiotic fixation. This is unlikely to be the case when the legume has access to soil N (McAuliffe et al. 1958). Also this method does not include fixed N_2 that has been

released into the soil by death and decay of legume material during the growth period.

Improved estimates of N_2 fixation have been obtained by analysing the soil and herbage N changes over time (Sears et al. 1965; Agboola and Fayemi 1972; Brock 1973). However, it is difficult to obtain an accurate estimate of changes in soil N because the changes are generally small relative to the amount of total N present in soils. For example, a 5% error in the estimation of total soil N in the study by Brock (1973) was equivalent to 160 kg N ha^{-1} or approximately two-thirds of the annual estimate of N fixed by suckling clover. The accuracy can be improved by collecting a large number of samples or by measuring the changes over a long period (Goh et al. 1978). Thus, N_2 fixation studies involving measurement of changes in legume + soil N with time have commonly lasted several years (Brock 1973; Simpson 1976).

Nitrogen balance techniques have also been employed to determine N_2 fixation (Greenland 1977; Knowles 1980; Herridge 1982c). These techniques require the quantification of all other inputs and outputs of N and may be summarised in the following equation (Greenland 1977; Herridge 1982c):

$$F_L = \Delta N + C + L + V + E - F_N - M - A - DR \qquad (1)$$

where F_L = rate of symbiotic N_2 fixation;

ΔN = change in total soil N with time;

C = harvested legume N (grain and vegetation);

L = N lost through leaching;

V = gaseous losses of N by denitrification, burning and ammonia volatilization;

E = N removed with eroded soil;

F_N = rate of non-symbiotic N_2 fixation;

M = N added in fertilizers, seed and manures;

A = N added via ammonia absorption by plants; and

DR = N added in dust and rainfall.

The terms E, F_N, M, A and DR are usually small and have often been omitted in N balance studies (Greenland 1977).

With N balance studies on legume/grass pastures, a term for the fixed N_2 of the legume that has been transferred to the associated grass (see section 2.2.3.1) should also be added to the right-hand side of equation 1. In a N balance study in the field, Simpson (1976) grew subterranean clover, white clover and lucerne, each in association with cocksfoot, and estimated that the amount of N fixed over a three year period was 741, 1422 and 1532 kg N ha^{-1} respectively by measuring the amount of N removed in the legume tops, the increase in soil N (0-100 mm) and the N transferred from the legume to the associated grass. No allowance was made for N that was lost from the plant/soil system during this study. In fact, there have been no N balance studies made on pasture legumes which have included all of the components of equation 1, although individual components have been examined in detail. The accuracy of the N balance technique will be governed by the accuracy in measuring each of the components of the system.

The major assumption in estimating N_2 fixation by all total N accumulation methods, including N balance, is that the legume derives all of its N from atmospheric N_2. This is generally incorrect under field conditions because legumes will also utilize plant-available soil N (Domenach et al. 1979; Kohl et al. 1980).

2.3.2 Difference methods

The total N accumulation methods can be improved by estimating the contribution of soil N to the total N assimilated by the legume and correcting the estimate of N fixed accordingly. This contribution has been assessed by growing a non-N_2-fixing reference plant in the same soil as that of the N_2-fixing legume. It is assumed that the legume and reference plant assimilate the same amount of soil N during the measurement period (Bell and Nutman 1971; Vest 1981) and that the amount of N fixed by the legume can be estimated from the equation,

$$N \text{ fixed by legume} = \text{Total } N_{\text{legume}} - \text{Total } N_{\text{reference plant}} \quad (2)$$

This procedure is referred to as the difference method (Williams et al. 1977). Three distinct versions of this method have been used to provide a correction for the soil N assimilated by the legume. These different versions arise through the choice of the non-N_2-fixing reference plant which are i) a non-nodulated legume, ii) an uninoculated legume, and iii) a non-legume.

2.3.2.1 Legume/non-nodulating legume

Non-nodulated legumes are considered to be the ideal reference plant for the nodulated legume of the same species because it is assumed that they have the same growth patterns, root morphologies and N uptake patterns (Vose et al. 1982). The two plants are normally grown concurrently in separate plots.

Soybean genotypes with a non-nodulating trait have been isolated (Weber 1966), and a recent report (Chalk et al. 1983) indicates that non-nodulating isolines of some cultivars of peanuts (Arachis hypogaea L.) and lucerne are now also available. However, it has been found that the root morphologies of nodulated and non-nodulated soybeans differ (Weber 1966; Vest 1971), and that nodulated soybeans explore a greater soil volume and extract more soil N than

non-nodulated soybeans (Ruschel et al. 1979). The greater uptake of soil N by the nodulated soybeans than the non-nodulated soybeans results in an overestimate of N_2 fixation by the nodulated soybeans.

2.3.2.2 Legume/uninoculated legume

An uninoculated legume of the same variety as the N_2-fixing plant can be used as a reference plant in the same way as the non-nodulating legume (section 2.3.2.1). For this method to work it is necessary that the soil on which the legumes are grown should contain no rhizobium species capable of establishing an effective N_2-fixing system with the legume. Alternatively, the reference plant can be inoculated with an ineffective N_2-fixing strain of rhizobia to minimize nodulation by effective strains. In both cases, the test legume is inoculated with an effective N_2-fixing rhizobium strain, in a separate plot to the reference plant. It is apparent that great care must be taken to avoid contamination of the reference plots.

These methods have been used in the field with crop (Bezdicek et al. 1978; Amarger et al. 1979) and pasture (Bell and Nutman 1971; Williams et al. 1977) legumes but they obviously suffer from similar drawbacks as the method which employs a non-nodulating legume as the reference plant. However, in contrast to the findings of Ruschel et al. (1979) that non-nodulated soybeans absorbed less soil N than nodulated soybeans (see section 2.3.2.1), Amarger et al. (1979) found that uninoculated soybeans assimilated more soil N than nodulated soybeans. These results of Amarger et al. (1979) caused an underestimation of N_2 fixation by the nodulated soybeans. Similarly, Williams et al. (1977) measured a 46% underestimation of N_2 fixation by nodulated subterranean clover when uninoculated subterranean clover was used as the reference plant due to greater assimilation of soil N by the uninoculated plant.

A major disadvantage with this method, particularly with pasture legumes, is the inability to ensure that the soils are free from effective N_2-fixing rhizobia, especially where the legumes are routinely grown (Bell and Nutman 1971).

2.3.2.3 Legume/non-legume

Non-legumes can also be used as reference plants and are grown concurrently in separate plots adjacent to the crop legumes (Richards and Soper 1979) or in the same plot as the pasture legumes (Wagner 1954). For this method to work it is essential that the legume and non-legume assimilate the same amount of soil N even though there may be differences in root morphologies, growth patterns, etc. It is also important that the N assimilated by the non-legume comes from the soil only i.e. that there is no N_2 fixation by free-living bacteria associated with the non-legume (see section 2.4.3.4).

Richards and Soper (1979) found that barley extracted the same amount of soil N as fababeans (Vicia faba L.), resulting in an accurate estimate of N_2 fixation by this method. In contrast, Williams et al. (1977) measured a 41% underestimation of N_2 fixation by purple vetch (Vicia benghalensis L.) when barley was used as the reference plant, due to greater uptake of soil N by barley.

Bell and Nutman (1971) grew nodulated lucerne and used several reference plants including uninoculated lucerne, lucerne inoculated with ineffective N_2-fixing rhizobium and ryegrass. The found that ryegrass generally assimilated a similar amount of soil N as nodule-free lucerne whereas the ineffectively nodulated lucerne often extracted more soil N. The uninoculated lucerne became contaminated by indigenous rhizobium thereby making it useless as a reference plant. The major problem with the N difference methods, whether

using non-nodulated legumes, uninoculated legumes or non-legumes as reference plants, is to ensure that the legume and reference plant assimilate the same amount of soil N.

In reviewing the N difference methods, Hardy and Holsten (1977) noted that they generally underestimate N_2 fixation since the legume utilizes less soil N than the reference plant. Furthermore, they concluded that they may not even provide a valid relative comparison of N_2 fixation between sites or plant varieties.

2.4 ^{15}N methods

Increasing use is being made of the stable isotope, ^{15}N, in studies of N_2 fixation by legumes. This isotope occurs in the atmosphere at a constant abundance (Mariotti 1983) of 0.3663 ± 0.0004 atoms % ^{15}N (Junk and Svec 1958). The remaining N (i.e. 99.6337%) occurs as ^{14}N. Although the chemical properties of ^{15}N and ^{14}N are almost identical, the small differences in mass and activation energies can cause changes in the isotopic composition during N cycling in the soil (see section 2.4.4) and as a consequence the isotopic composition of soil N is different from that of atmospheric N_2. It is possible to use this difference in natural abundance of ^{15}N between atmospheric N_2 and soil N to estimate N_2 fixation by legumes (Amarger et al. 1979; Bergersen and Turner 1983). The sensitivity of this method can be increased by artificially enriching the atmosphere or soil with ^{15}N (Hauck and Bremner 1976; Bergersen 1980; Vose et al. 1982). From a knowledge of the isotopic composition of N derived from the atmosphere and the soil, and the measurement of the isotopic composition of the legume N, an estimate can be made of the proportion of legume N derived from these two sources.

The main factors limiting the widespread use of ^{15}N techniques for measuring N_2 fixation have been the high costs of ^{15}N-labelled compounds and of the mass spectrometer required for ^{15}N analysis (Bremner 1977).

2.4.1 $^{15}N_2$ method

The use of a $^{15}N_2$-labelled atmosphere to estimate N_2 fixation has been the primary standard for comparison with other methods (Burris 1974; Bergersen 1980). With this method, the test legume is exposed to a $^{15}N_2$-enriched atmosphere in a gas-tight chamber for an appropriate period of time. The plant material and the atmosphere are then analysed for ^{15}N and the proportion (P) of legume N obtained from the atmosphere is calculated from the relationship (Vose et al. 1982):

$$P = \frac{\text{Atoms \% } ^{15}\text{N excess}_{\text{legume}}}{\text{Atoms \% } ^{15}\text{N excess}_{\text{atmosphere}}} \qquad (3)$$

where atoms % ^{15}N excess = atoms % $^{15}N_{sample}$ - atoms % $^{15}N_{control}$; in this case the control is unenriched atmospheric N_2 and therefore atoms % $^{15}N_{control}$ = 0.3663 (Bergersen 1980).

This method has been employed commonly to confirm the presence of a N_2-fixing system in legumes or associated with non-legumes (e.g. Burris 1974; De-Polli et al. 1977; Ruschel et al. 1979) and to establish a C_2H_2/N_2 conversion factor for use in acetylene reduction assays (section 2.2.2) (e.g. Hudd et al. 1980; Saito et al. 1980).

Ross et al. (1964) described a gas-tight growth chamber for use in $^{15}N_2$ studies and stressed the importance of minimising changes in the gaseous composition of the atmosphere and of following the changes in the isotopic composition of the $^{15}N_2$-labelled atmosphere

with time. Obviously, such a procedure is expensive to establish and difficult to maintain. Its complexity limits the use of this method to the study of only a few plants (De-Polli et al. 1977).

Both the $^{15}N_2$ method and the acetylene reduction method (section 2.2.2) utilize a gas-tight chamber in which the legume is incubated and therefore they are subject to the same problems. The difficulty in maintaining a constant atmospheric composition and in simulating environmental conditions that pertain to the field, mean that both methods can only be used for short periods. This necessitates the measurement of variations in N_2 fixation with time to obtain an integrated long-term estimate of N_2 fixation. Thus, the $^{15}N_2$ method is not considered to be a suitable method for estimation of N_2 fixation by crop or pasture legumes in the field (Vose et al. 1982).

2.4.2 A-value method

The A-value method for estimating N_2 fixation was proposed by Fried and Broeshart (1975) and involves the application of ^{15}N-labelled fertilizer to a legume and reference plant growing in the same soil. With this method, a reference plant is used to obtain an integrated measure of the isotopic composition of the fertilizer + soil N that is assimilated by the legume during its growth period. This contrasts with the N difference methods (section 2.3.2), where a reference plant is used to measure the amount of soil N assimilated by the legume. Thus, with the A-value method, the legume and reference plant do not have to assimilate the same amount of soil N.

The A-value is defined as the amount of available N in the soil in units of a fertilizer standard (Fried and Broeshart 1975) and is expressed as

$$A = F\ (1-f)/f \qquad (4)$$

where F is the amount of fertilizer N added and f is the proportion of N in the plant derived from the fertilizer N. The value of f is obtained from the equation

$$f = \text{atoms \% } {}^{15}\text{N excess}_{plant} / \text{atoms \% } {}^{15}\text{N excess}_{fertilizer}. \qquad (5)$$

Separate A-values can be determined for the legume and the reference plant, designated $A_{soil + atmosphere}$ for the legume and A_{soil} for the reference plant. With the legume, it is assumed that the isotopic composition of N derived from the soil and the atmosphere are both equal to 0.3663 atoms % ^{15}N. Although this is unlikely to be correct (see section 2.4.4), any small differences are unimportant if the fertilizer has a high ^{15}N enrichment.

The amount of legume N derived from the atmosphere can be calculated from the expression (Fried and Broeshart 1975):

$$\text{kg N fixed ha}^{-1} = [A_{atmosphere + soil} - A_{soil}] \times [\%\text{ of F recovered by the legume}/100]. \qquad (6)$$

where the A-values are also expressed in kg N ha^{-1}.

This can be rewritten as

$$\text{kg N fixed ha}^{-1} = [A_{legume} - A_{reference\ plant}] \times \frac{f_{legume} \times \text{legume N (in kg N ha}^{-1})}{F_{legume}\ (\text{in kg N ha}^{-1})} \qquad (7)$$

The assumptions inherent in this method are essentially the same as those for the ^{15}N isotope dilution method and these are discussed in section 2.4.3. It was believed that the A-value method had the advantage that the legume and reference plant could be supplied with different rates of fertilizer N without affecting the determination of N_2 fixed, because it was assumed that the A-value did not vary with rate of N application. However, many workers have measured changes in A-values with changes in the rate of N application (Deibert et al. 1979; Rennie 1979; Ruschel et al. 1979). This has generally been attributed to changes in N fertilizer availability (e.g. through losses of fertilizer N by denitrification or volatilization) or to changes in soil N supply (e.g. through changes in root extension).

The A-value method can only be used when N fertilizer is applied and this may affect the fixation of N_2 by the legume (section 2.4.3.1). In order to avoid such effects on N_2 fixation, low levels of N application are normally used and it is under these conditions that A-values are estimated with the least precision (Rennie 1979).

To estimate the proportion of legume N derived from atmospheric N_2 with the A-value method it is necessary to divide the kg N fixed ha^{-1} (from equation 7) by the total N yield of the legume. Thus, it is yield-dependent and its precision can never exceed that of other yield-dependent methods (Vose et al. 1982).

2.4.3 ^{15}N isotope dilution method

The A-value concept was used in the development of a ^{15}N isotope dilution method for estimating N_2 fixation by legumes (Fried and Middelboe 1977). When this method is used, ^{15}N-labelled fertilizer or ^{15}N-labelled organic material is also added to soil in which a legume and reference plant are growing. However, in

contrast to the A-value method, in the ^{15}N isotope dilution method the same rate of N application is used with the two plant species. The ^{15}N-labelled soil N absorbed by the legume is diluted by N_2 from the atmosphere, thereby invoking the term isotope dilution which has commonly been used to describe this method (Rennie et al. 1978; Edmeades and Goh 1979; Rennie 1979; Heichel et al. 1981a; Knowles 1981).

The following section describes how the proportion (P) of legume N obtained from atmospheric N_2 can be determined (Fried and Middelboe 1977): If f_1 and f_2 are the proportions of plant N derived from the added N by the reference plant and legume respectively; and S_1 and S_2 are the proportions of plant N derived from the soil by the reference plant and legume respectively;

then

$$f_1 + S_1 = 1 \qquad (8)$$

$$f_2 + S_2 + P = 1. \qquad (9)$$

The major assumption inherent in the ^{15}N isotope dilution method (and the A-value method) is that the legume and reference plant absorb the same relative amounts of N from the added N and soil N, i.e.

$$f_1/S_1 = f_2/S_2. \qquad (10)$$

Therefore

$$S_2 = S_1 \times f_2/f_1. \qquad (11)$$

If S_1, from equation 8, is substituted into equation 11, then

$$S_2 = (1-f_1) \times f_2/f_1 = (f_2/f_1)-f_2. \qquad (12)$$

Rearranging equation 9 to give

$$P = 1-f_2-S_2, \qquad (13)$$

and substituting for f_2 and S_2 from equations 5 and 12, then

$$\underline{P} = 1 - \frac{\text{atoms \% } ^{15}\text{N excess}_{\text{legume}}/\text{atoms \% } ^{15}\text{N excess}_{\text{added N}}}{\text{atoms \% } ^{15}\text{N excess}_{\text{reference}}/\text{atoms \% } ^{15}\text{N excess}_{\text{added N}}} \qquad (14)$$

Therefore

$$\underline{P} = 1 - \frac{\text{atoms \% } ^{15}\text{N}_{\text{excess}_{\text{legume}}}}{\text{atoms \% } ^{15}\text{N excess}_{\text{reference}}} \cdot \qquad (15)$$

In this case, atoms % ^{15}N excess = atoms % ^{15}N - B, where B = atoms % ^{15}N of legume N derived entirely from atmospheric N_2 and is commonly taken as 0.3663. Therefore, equation 15 can be written as

$$\underline{P} = \frac{\text{atoms \% } ^{15}\text{N}_{\text{reference}} - \text{atoms \% } ^{15}\text{N}_{\text{legume}}.}{\text{atoms \% } ^{15}\text{N}_{\text{reference}} - \quad \text{B}} \qquad (16)$$

Thus, the estimate of $\underline{P}$ is independent of the dry matter or N yield of the legume. The yield of fixed N_2 is obtained by multiplying $\underline{P}$ by the total N yield of the legume.

The ^{15}N isotope dilution method has been extensively used to determine $\underline{P}$ and the amount of N fixed by nodulated soybeans in field studies using, as reference plants, soybeans that were uninoculated,

inoculated with ineffective N_2-fixing rhizobium or non-nodulated (Domenach et al. 1979; Rennie 1982; Rennie et al. 1982; Talbott et al. 1982; Patterson and LaRue 1983). The estimates of P obtained for nodulated soybeans by this method were similar to those obtained using other methods (Table 2.2). Patterson and LaRue (1983) also obtained similar estimates of P with ^{15}N isotope dilution and N difference methods but obtained a much lower estimate when the acetylene reduction method was used (Table 2.2). They attributed the latter result, in part, to incomplete recovery of nodules.

The ^{15}N isotope dilution method has been used in the field to determine the amount of N fixed by pasture legumes in U.S.A. (Phillips and Bennett 1978; West and Wedin 1981; Heichel et al. 1981b), New Zealand (Goh et al. 1978; Edmeades and Goh 1979; Steele and Shannon 1982), Scotland (Haystead and Lowe 1977; Haystead and Marriott 1978) and Australia (Vallis et al. 1977; Bergersen and Turner 1983), but there have been few studies comparing it with other methods for estimating N_2 fixation (Table 2.3). Phillips and Bennett (1978) obtained lower estimates of the amount of N fixed by subterranean clover using the acetylene reduction method than they did with the ^{15}N isotope dilution method (Table 2.3). A similar comparison of methods by Goh et al. (1978) with white clover revealed that the relative difference between methods varied with the length of time of incubation of samples in the acetylene reduction method (Table 2.3). Goh et al. (1978) and Phillips and Bennett (1978) considered that the ^{15}N isotope dilution method gives the most accurate, long-term, quantitative estimate of N_2 fixation by field legumes but that the assumptions inherent in the method should be validated.

Table 2.2. Effect of method on the estimate of the percentage of nitrogen fixed from the atmosphere by crop legumes.

Legume	Reference plant	Method: Acetylene reduction	Nitrogen difference	A value	^{15}N I.D.[1]	Natural abundance	
soybean	nn.[2] soybean				58	64	Domenach et al. 1979
soybean	ray-grass				62	65	
soybean	nn. soybean		54			44	Ruschel et al. 1979
soybean	nn. soybean				29	27	
soybean	nn. soybean		34			14	Kohl et al. 1980
soybean	nn. soybean		56			54	
soybean	nn. soybean	25	78		66		Patterson & LaRue 1983
navybean[3]	un.[4] navybean		5			33	Rennie 1979
navybean	un. navybean		13	28	13		

[1] Isotope dilution; [2] Non-nodulated; [3] *Phaseolus vulgaris* L.; [4] Uninoculated.

Table 2.3. Effect of method on the estimate of the amount (kg N ha^{-1}) of nitrogen fixed from the atmosphere by field-grown pasture legumes.

Legume	Reference plant	Method: Acetylene reduction	Nitrogen difference	A value	^{15}N I.D.[1]	Natural abundance	
sub.[2] clover	un.[3] sub. clover		50	93			Williams et al. 1977
sub. clover	Bromus mollis	57			103		Phillips and
sub. clover	Bromus mollis	89			183		Bennett 1978
white clover	ryegrass	6.4[4]			4.9		
white clover	ryegrass	4.1[5]			4.9		Goh et al. 1978
white clover	ryegrass	3.8[6]			4.9		
white clover	ryegrass				(85)[7]	(45)	Edmeades & Goh 1979
white clover	ryegrass				(91)	(62)	
sub. clover	ryegrass				21	14	Bergersen & Turner
sub. clover	ryegrass				47	48	1983

[1]Isotope dilution; [2]Subterranean; [3]Uninoculated; [4-6]1,3 and 6 hours incubation, respectively; [7]Values are from % N fixed from the atmosphere (amounts of fixed N were not presented).

The various assumptions associated with this method are discussed in sections 2.4.3.1 to 2.4.3.5.

2.4.3.1 Effect of nitrogen application on N_2 fixation

In order to use the ^{15}N isotope dilution method to estimate N_2 fixation it is necessary to amend the soil with ^{15}N-labelled fertilizer or ^{15}N-labelled organic material so that its dilution by atmospheric N_2 can be measured. It is important that the amount of N added should have no effect on the amount of N fixed by the legume under study.

Munns (1977) reviewed the effect of N addition on N_2 fixation and noted that it depends on the stage of growth of the legume, the host species and rhizobial strain, and the form and concentration of added N. Most experiments have been conducted soon after sowing the legume and an increase in yield and N_2 fixation have often been measured at low rates of N application, due to increased root growth and enhanced nodulation (Richardson et al. 1957; Allos and Bartholomew 1959; Pate and Dart 1961; Dart and Wildon 1970; Bethlenfalvay et al. 1978). However, the yield advantage generally disappears once the plants have become established (Fishbeck and Phillips 1981). Less attention has been given to the effect on established legumes, particularly at the low rates of N commonly used in ^{15}N isotope dilution experiments.

In a pot experiment, Vance and Heichel (1981) found no effect of N addition on the yield of lucerne but obtained a decrease in the estimate of P at rates of N application of 40 and 80 kg ha^{-1}. McAuliffe et al. (1958) grew legumes together with grass in pots with increasing levels of N application. Although legume yield and total N yield were unaffected by rates up to 225 kg N ha^{-1}, P declined as the rates of N addition were increased from 28 to 225 kg N ha^{-1}.

In a field experiment with mixed white clover/ryegrass pasture, Ledgard and Saunders (1982) measured no effect of 25 to 100 kg N ha^{-1} on clover N yield for the first two harvests after N application, whereas there were large increases in grass N yield. However, in subsequent harvests, there was a decrease in clover N yield due to increased competition from the grass. They measured a decrease in the level of acetylene reduction by clover at 100 kg N ha^{-1}, initially because of substitution for uptake of fertilizer N and subsequently because of reduced clover yields. Thus, in a mixed legume/grass pasture, added N can affect N_2 fixation directly, possibly due to depletion in supply of photosynthate to the nodules (Manhart and Wong 1980), and indirectly due to changes in the proportion of pasture components.

The rate of application of ^{15}N-labelled fertilizer in ^{15}N isotope dilution studies in the field has varied from < 1 kg N ha^{-1} (Goh et al. 1978) to 14 kg N ha^{-1} (Heichel et al. 1981a) with pasture legumes and up to 27 kg N ha^{-1} with crop legumes (Patterson and LaRue 1983). One might suspect that the rate of N application in some of these experiments was sufficiently high to cause an incorrect estimate of P. Talbott et al. (1982) used 134 kg N ha^{-1} and immobilised it by adding large amounts of sucrose. Although this may not have directly affected N_2 fixation, it will have affected the soil biomass and possibly induced changes in the plant-available soil N status resulting in an indirect effect on N_2 fixation (Vose et al. 1982). Thus, the application of ^{15}N-labelled materials can have a direct or indirect effect on N_2 fixation and therefore these effects should be minimised by using low rates of N addition and/or by monitoring these effects in an associated study.

2.4.3.2 Ratio of uptake of added nitrogen and soil nitrogen

When a ^{15}N-labelled compound is used to label the soil N, it is assumed that the legume and reference plant will absorb added N and soil N in the same ratio, irrespective of the amounts absorbed (McAuliffe et al. 1958). Vallis et al. (1967) suspected that this assumption may have been invalid in a pot experiment with Townsville lucerne (Stylosanthes humilis H.B.K.) and Rhodes grass (Chloris gayana Kunth.), but they noted that it would be difficult to prove. Wagner and Zapata (1982) attempted to test this assumption with soybeans and fababeans by measuring the relative uptake of labelled and soil sulphur and assumed that it would be the same as that for added and soil N. Their study revealed that the ratio of uptake of labelled and soil sulphur was similar for various legume/non-legume combinations but found that some plants differed in their relative absorption of N and sulphur. No suitable test for this assumption has yet been devised.

2.4.3.3 Importance of matching the legume and reference plant

An accurate measure must be made of the isotopic composition of the N assimilated from the added N + soil N by the legume during the measurement period after addition of a ^{15}N-labelled compound. Techniques involving chemical extraction of soil N could be used but they may not measure the pool which is available to the plant (Stanford 1982). However, this technique was used successfully by Chalk et al. (1983) in pot experiments, where ^{15}N-labelled soil organic matter was used. Where ^{15}N-labelled fertilizer is used, the isotopic composition of the added N + soil N changes considerably with

time (Witty 1983b) and therefore many extractions may be needed to integrate these changes over the measurement period. Thus, a non-N_2-fixing reference plant is commonly used to obtain an integrated estimate of the isotopic composition of N derived from the fertilizer + soil by the legume. This estimate may not be correct if (i) the legume and reference plant differ in their uptake of N from different depths in the soil and this coincides with differences in the isotopic composition of plant-available N with soil depth (Phillips and Bennett 1978; Edmeades and Goh 1979), or (ii) the legume and reference plant differ in their pattern of growth with time and this is associated with changes in the isotopic composition of plant-available N with time (Witty 1983a,b).

Variations in the ^{15}N concentration of soil N with depth probably occur in most field experiments because ^{15}N-labelled compounds are commonly applied to the soil surface in order to avoid disturbance of the soil (Knowles 1980). Thus, it is thought to be important to match the legume and reference plant in their root distribution, or zone of N uptake, with depth in the soil so that they have the same ratio of uptake of added N and soil N (see section 2.4.3.2).

Soil N is commonly labelled with ^{15}N by a single application of ^{15}N fertilizer at the start of the growth period (e.g. Phillips and Bennett 1978; Domenach et al. 1979; Heichel et al. 1981a). This is likely to result in a decrease with time in the ^{15}N concentration of N derived from the fertilizer + soil due to removal of plant-available ^{15}N by plant uptake, leaching, denitrification and immobilization and to the dilution of plant-available ^{15}N by unenriched N released by mineralization. If this coincides with differences between the legume and reference plant in their pattern of N assimilation with time, then

an incorrect estimate of the amount of N fixed by the legume can result (Witty 1983a,b). Thus, where there is a change in the isotopic composition of plant-available soil N with time it is also important to match the legume and reference plant in their patterns of N assimilation with time, irrespective of their growth rates.

It has been suggested that a non-nodulated legume, of the same species as the nodulated legume being studied, would be the ideal reference plant (see section 2.3.2.1). However, Patterson and LaRue (1983) found that the ^{15}N concentration of nodulated soybeans often exceeded that of the non-nodulated isoline, resulting in negative estimates of P. They suggested that this result may be due to differences in root growth between the two plants.

Few published studies with the ^{15}N isotope dilution method have used more than one reference plant. Domenach et al. (1979) obtained similar estimates of P for nodulated soybeans when non-nodulated soybeans and ray-grass were used as reference plants (Table 2.2). However, Rennie (1982) found that the use of uninoculated or ineffectively inoculated soybeans and barley as reference plants gave similar estimates of P for nodulated soybeans, whereas a higher P value was obtained when non-nodulated soybean was used as a reference plant. Similarly, Witty (1983b) found that the reference plants, oilseed rape (Brassica rapa L.) and ryegrass, gave similar estimates of the amount of N fixed by fababeans, french beans (Phaseolus vulgaris L.), peas (Pisum sativum L.) and red clover, whereas estimates were lower when barley was used as a reference plant. This was attributed to differences in the pattern of growth by barley with time relative to the other plants. However, he did not examine differences with depth in the plant root distribution and the isotopic composition of soil N.

In all of these studies, the reference plants were grown in plots separate from the legumes. This contrasts with pastures, where the legumes are commonly grown in association with grasses (which are used as the reference plant) and both plants compete for the same source of ^{15}N-labelled plant-available soil N. Therefore the effect of different reference plants on the estimation of P by pasture legumes grown together with grasses may be different from that obtained where the legumes and reference plants are grown separately. However, there have been no published studies on the effect of different reference plants on the estimation of P in mixed legume/grass pastures.

2.4.3.4 N_2 fixation associated with the reference plant

When a reference plant is used to sample the plant-available soil N it is important that no N_2 fixation is directly associated with the reference plant. Otherwise the reference plant would have a lower ^{15}N concentration than if all of its N was derived from the fertilizer + soil, and N_2 fixation by the legume would be underestimated. With uninoculated legumes or legumes nodulated with ineffective N_2-fixing rhizobium as reference plants, there is always the concern that these plants may become effectively nodulated through contamination by added or indigenous rhizobium (Bell and Nutman 1971). Even when non-legumes are used as reference plants, their roots can greatly enhance the activity of the free-living N_2-fixing bacteria which are present in most soils (Neyra and Dobereiner 1977; van Berkum and Bohlool 1980). If this fixed N_2 is assimilated by the non-legumes, N_2 fixation by the legume under study will again be underestimated.

Use of the acetylene reduction method has revealed high nitrogenase activity associated with numerous tropical grasses (Day et

al. 1975b; Dobereiner and Day 1975; Bulow and Dobereiner 1975) and this has been confirmed by measuring the incorporation of ^{15}N into non-legumes from a $^{15}N_2$-labelled atmosphere (De-Polli et al. 1977). In Australia, Weier (1980) measured acetylene reduction activity associated with three tropical grass species, and Weier et al. (1981) estimated Panicum maximum L. to fix 2.7 kg N $ha^{-1}yr^{-1}$ in a tropical climate.

In temperate climates, only a limited number of grass species have been investigated for nitrogenase activity and it has been found that activity has been low, especially for species with a C-3 photosynthetic system (Tjepkema and Burris 1976; Neyra and Dobereiner 1977). Vlassak et al. (1973) estimated that native grassland in Canada fixed about 1 kg N $ha^{-1}yr^{-1}$, while Steyn and Delwiche (1970) found that a number of Californian grasslands averaged 2.5 kg N $ha^{-1}yr^{-1}$. In a comparison of temperate grass species, Nelson et al. (1976) measured the highest acetylene reduction activity with Agrostis tenuis Sibth. (74 g N fixed $ha^{-1}day^{-1}$), while most other species, including ryegrass (5 g N fixed $ha^{-1}day^{-1}$), were much less active. In Australia, Thompson et al. (1984) examined a range of temperate and tropical grasses in northern New South Wales and measured a maximum activity in the field equivalent to 1.9 g N fixed $ha^{-1}hr^{-1}$. They concluded that it was of no agronomic significance. However, the contribution of fixed N_2 to the non-legumes may be sufficient to affect the estimate of N_2 fixation by legumes using the ^{15}N isotope dilution method. There have been no studies on the measurement of N_2 fixation by legumes with the ^{15}N isotope dilution method that have also examined for N_2 fixation associated with the reference plant.

2.4.3.5 Transfer of fixed N_2 from legume to reference plant

When the ^{15}N isotope dilution method is used to estimate N_2 fixation in mixed legume/grass pastures it is also assumed that there is no direct transfer of fixed N_2 from the legume to the reference plant during the measurement period. If this were to occur, the ^{15}N concentration of the reference plant would be less than if all of its N was derived from the fertilizer + soil, and N_2 fixation by the legume would be underestimated.

In intensively grazed, legume/grass pastures most of the N_2 fixed by the legume is returned to the soil via the grazing animals as dung and urine (Sears 1953) and some of this can then be assimilated by grasses. This source of error can be obviated by excluding grazing animals during the measurement period (Goh et al. 1978; Steele and Shannon 1982). Another possible source of error is that of overground transfer which may involve the direct loss of ammonia (NH_3) from the leaves of the legume and the subsequent absorption of this NH_3 by the grass, or vice versa. This possibility was reviewed by Farquhar et al. (1983) who noted that plants grown with ^{15}N-enriched fertilizer would tend to lose $^{15}NH_3$ and gain $^{14}NH_3$, even if the net flux of NH_3 was zero. Thus, transfer of NH_3 between plants in a mixed legume/grass pasture could lead to differential changes in their isotopic composition. Farquhar et al. (1983) concluded that the magnitude of gaseous N transfer, and its importance to N yield, is uncertain. However, it is likely to be small (G.D. Farquhar, pers. comm.).

'Underground' transfer of fixed N_2 from the legume to the reference plant can also occur and this may involve at least two

different mechanisms. Nitrogen may be excreted from intact roots of growing legumes (Virtanen et al. 1937) and then be absorbed by the roots of the associated reference plant. However, the main mechanism for underground transfer involves the decomposition of legume roots and nodules (Butler et al. 1959) and absorption of the released N by the reference plant. Nitrogen released from decaying roots and nodules enters the soil N pool and is equally available for uptake by both the legume and reference grass. Thus, isotope dilution measurements of N_2 fixation are not necessarily invalidated by this phenomenon. Haystead and Marriott (1979) found that much of the N released from clover roots and nodules was immobilized by soil bacteria and therefore not available for uptake by either plant. Jansson (1971) also noted that heterotrophic soil microflora were considerably more competitive for uptake of mineralized ammonium than plants.

Numerous pot experiments using ^{15}N-labelled fertilizer have demonstrated negligible transfer of fixed N_2 from legumes to associated non-legumes for periods up to one year (Henzell 1962; Simpson 1965; Vallis et al. 1967; Broadbent et al. 1982). In the field, N transfer has generally been measured over relatively long periods of time with grazing animals being excluded. Simpson (1976) measured cumulative N transfer over three years from subterranean clover, white clover and lucerne to a companion grass of 20, 6 and 3% of the total N fixed, respectively. Similarly, Johansen and Kerridge (1979) obtained 12 to 17% N transfer from a range of tropical legumes to an associated grass over a five year period. In contrast, Broadbent et al. (1982) found no transfer of fixed N_2 from white clover to ryegrass over a six month period but subsequently

measured considerable transfer (up to 79%), after about 18 months. From this result, they concluded that ^{15}N isotope dilution methods were unsuitable for measuring N_2 fixation in legume/grass mixtures. However, they overlooked the fact that ^{15}N isotope dilution measurements in pastures are usually made over relatively short periods (commonly less than six months) and that longer term measurements are made by integrating the results from several of these periods (Edmeades and Goh 1978; Steele and Shannon 1982; Bergersen and Turner 1983). During a ^{15}N isotope dilution study over a 17 week period, Haystead and Marriott (1978) measured less than 6% transfer of fixed N_2 from white clover to an associated grass. Thus, it is commonly considered that N transfer (except via grazing animals) is minimal in the short term, being related to legume production in the previous year rather than to current legume growth (Simpson 1976; Haystead and Marriott 1978).

2.4.3.6 Distribution of ^{15}N between plant parts

Differences in the concentration of ^{15}N between plant parts following uptake of a ^{15}N-labelled compound may affect the estimate of P if only a portion of the plant (e.g. shoots) is sampled for analysis. The distribution of ^{15}N between plant parts may be affected by the unlabelled plant N present at the time of application of ^{15}N-labelled compound. If the whole plant is not sampled then it is important that the newly accumulated N, whether fixed or absorbed from the soil, should equilibrate with the plant N present at the start of the measurement period (Haystead and Lowe 1977).

Smith and Silva (1969) used total N analysis of roots, crowns and shoots to illustrate that organic N reserves are mobilized to the

new foliage during plant regrowth. This was examined in detail with ^{15}N by Phillips et al. (1983) using subterranean clover and Bromus mollis L. They found that the amount of N recycled through the crowns and roots of these plants during a period between three shoot harvests was 1.0 to 1.4 times the average amount of N present in these plant parts. Therefore, the rapid cycling of N between plant parts will minimise differences in the isotopic composition of N between plant parts.

Haystead and Lowe (1977) found that the ^{15}N concentration of N in shoots and roots was the same for white clover and ryegrass by 41 days after ^{15}N application, indicating that there was no need to harvest the whole plant to obtain on accurate estimate of P. In contrast, Rennie et al. (1978) measured some variation in the isotopic composition of plant parts of nodulated and non-nodulated soybeans grown at three different locations, but obtained different estimates of P using the different plant parts at only one of these locations.

Yield-dependent estimates of P have been proposed (Haystead and Lowe 1977; Bergersen and Turner 1983) so that only uptake of ^{15}N during the measurement period is used. Haystead and Lowe (1977) adjusted the plant enrichment of N at the end of the measurement period (atoms % ^{15}N $excess_2$), for N present in the legume and reference plant prior to ^{15}N application (N_1) by

$$\text{Atoms \% } ^{15}\text{N excess during the measurement period} = \frac{\text{Atoms \% } ^{15}\text{N excess}_2}{1 - (N_1/N_2)} \qquad (17)$$

They then used equation 15 to estimate P by using the adjusted ^{15}N enrichment (equation 17) for the legume and reference plant.

Bergersen and Turner (1983) obtained yield-dependent estimates of P by measuring the ^{15}N yields of the legume and reference plant at the start and end of the measurement period:

$$P = [100(r-q) - \Delta NX]/[\Delta N(B-X)] \quad (18)$$

where q and r are the ^{15}N yields of the legume at samplings 1 and 2 respectively; ΔN is the increment of total N by the legume during the measurement period; B is the atoms % ^{15}N of the legume when grown with atmospheric N_2 as the sole source of N; and X is obtained from the reference plant by:

$$X = 100\ (x-w)/\Delta N' \quad (19)$$

where w and x are the ^{15}N yields of the reference plant at samplings 1 and 2 respectively and $\Delta'N$ is the increment of total N by the reference plant during the measurement period.

These yield-dependent methods account for differences between the legume and reference plant in the dilution of ^{15}N assimilated during the measurement period by unenriched material present prior to ^{15}N addition, which could cause erroneous estimates of P by the conventional method of calculation (equation 16). However, when the plant enrichments at the end of the measurement period are very high relative to those at the start, this effect is likely to be negligible.

2.4.4 Natural ^{15}N abundance method

When natural differences in the isotopic composition between atmospheric and soil N occur, they can be used to estimate P in the same manner as the ^{15}N isotope dilution method (section 2.4.3). However these differences are small and therefore the expression $\delta^{15}N$ (in ‰) is commonly used (Rennie et al. 1978), where:

$$\delta^{15}N = \frac{(\text{atoms \% } ^{15}N_{sample} - \text{atoms \% } ^{15}N_{standard})}{\text{atoms \% } ^{15}N_{standard}} \times \frac{1000.}{1} \qquad (20)$$

The standard is usually atmospheric N_2 (Mariotti 1983) or unlabelled ammonium sulphate (Bergersen 1980).

Differences in the natural abundance of ^{15}N between atmospheric and soil N occur because almost all N transformations in the soil result in isotopic fractionation (Mariotti 1982). During denitrification (Delwiche and Steyn 1970; Blackmer and Bremner 1977) and volatilization (Haurat et al. 1981; Turner et al. 1983), fractionation can be relatively large, resulting in a preferential loss of the lighter isotope, ^{14}N, and an enrichment in ^{15}N of the residual N. The net effect of the soil N transformations and their associated isotopic fractionations is an increase in the ^{15}N abundance of soil N compared with atmospheric N_2 (see Table 2.4).

The ability to utilize the difference in natural abundance of ^{15}N between atmospheric and soil N for estimating N_2 fixation by legumes at a particular site will depend on the degree of spatial (both horizontal and vertical) variability in the ^{15}N abundance of soil N. Broadbent et al. (1980) reported a surprisingly large spatial variability in the $\delta^{15}N$ of total N from a range of surface soils, extending below and above that of the $\delta^{15}N$ of atmospheric N_2. They concluded that the natural ^{15}N abundance method could not be used for estimating N_2 fixation by legumes in the field. However, that is the only report of such large variations (see Table 2.4). In contrast, the $\delta^{15}N$ of total N in surface soils reported by others

Table 2.4. Isotopic composition of total nitrogen ($\delta^{15}N$, ‰) in surface soils.

Source	Mean	S.D.[1]	Range	No. soil samples
Australia				
Black and Waring (1977)	4.8	1.9	+ 2.9 to + 6.7	3
New Zealand				
Steele et al. (1981)	3.2	1.6	- 1.1 to + 6.8	61
Belgium				
Riga et al. (1970)	3.0	0.7	- 1.9 to 4.1	10
Canada				
Rennie and Paul (1975)	10.9	2.1	+ 6.1 to +12.9	11
Rennie et al. (1976)	8.8	1.2	+ 4.6 to +10.2	11
Karamanos et al. (1981)	9.4	3.0	+ 3.5 to +14.3	58
United States of America				
Cheng et al. (1964)	6.3	5.2	- 1.0 to +17.0	28
Bremner et al. (1966)	4.3	5.7	- 3.3 to +18.1	25
Bremner and Tabatabai (1973)	-0.2	2.1	- 4.4 to + 3.0	16
Shearer et al. (1974)	9.7	1.7	+ 6.8 to +13.3	19
Shearer et al. (1978)	9.2	2.1	+ 5.1 to +12.3[2]	139
Virginia (1980)	6.2	2.4	+ 2.5 to +10.1	11
Broadbent et al. (1980)	3.5	10.6	NOT REPORTED	196

[1] Standard deviation.

[2] Range within which 90% of the samples fell.

(Black and Waring 1977; Karamanos et al. 1981; Kohl et al. 1981) was different from that of atmospheric N_2 and showed small spatial variability, indicating that the natural abundance of ^{15}N can be used for field studies on N_2 fixation.

Variability in the natural abundance of ^{15}N in N from pasture soils subject to grazing by animals may be greater than that in cropping soils because it has been shown that the dung returned to the soil by grazing animals is enriched in ^{15}N compared with urine (Steele and Daniel 1978) and that dung and urine are voided separately onto relatively small areas (Ledgard et al. 1982).

Variations in the natural abundance of ^{15}N in total soil N have been measured within a soil profile, often reaching a maximum at depth (Delwiche and Steyn 1970; Virginia 1980; Steele et al. 1981). It is thought that plant-available soil N would also vary with soil depth, indicating that it may be important to match the legume and reference plant in their depth of root penetration, or zone of N uptake, so that they both assimilate soil N of the same ^{15}N abundance (Steele et al. 1981). This is synonymous with the corresponding assumption of the A-value and ^{15}N isotope dilution methods (see section 2.4.3.3).

Plant N derived from the soil is believed to be largely in the nitrate form and while this form may be less enriched in ^{15}N than the total N (compare Tables 2.4 and 2.5), it is still usually greater than that of atmospheric N_2. If the natural abundance of ^{15}N in plant-available soil N is different from that of atmospheric N_2, then it is possible to estimate P using an equation analagous to that of the ^{15}N isotope dilution method (equation 15), i.e.

Table 2.5. Isotopic composition of nitrate nitrogen ($\delta^{15}N$, ‰) in surface soils.

Source	Nitrate sampling[1]	Mean	S.D.[2]	Range	No. soil samples
Australia					
Black and Waring (1977)	I	2.4	2.3	-0.2 to + 4.2	3
Canada					
Rennie et al. (1976)	D	8.8	2.9	+2.7 to +11.0	9
United States of America					
Bremner and Tabatabai (1973)	I	-0.9	4.8	-9.6 to + 6.3	10
Feigin et al. (1974)	D	1.2	1.5	-2 to +10	12
Virginia (1980)	D	0.3	1.2	-0.8 to + 1.1	13

[1]Nitrate was either extracted directly (D) from the soil or following incubation (I).
[2]Standard deviation.

$$\underline{P} = 1 - \frac{\delta^{15}N_{legume}}{\delta^{15}N_{available\ soil\ N}}. \qquad (21)$$

The $\delta^{15}N_{available\ soil\ N}$ is commonly obtained from a non-N_2-fixing reference plant grown in the same soil as the legume.

The natural ^{15}N abundance method has been used qualitatively, to identify plants as being N_2-fixing or non-N_2-fixing (Virginia and Delwiche 1982), and quantitatively to estimate the amount of legume N fixed from atmospheric N_2. Amarger et al. (1979) grew inoculated and uninoculated soybeans and estimated N_2 fixation by the natural ^{15}N abundance method (i.e. from equation 21) and compared the results with those from the acetylene reduction and N difference methods. Estimates based on natural ^{15}N abundance were correlated with those from the acetylene reduction method but not with those from the N difference method. The different estimates between the natural abundance and N difference methods were due to differences in uptake of soil N by the inoculated and uninoculated soybeans, thus invalidating results from the N difference method.

Only two published comparisons of the natural ^{15}N abundance method with other methods for estimating N_2 fixation by pasture legumes have been made (see Table 2.3). Bergersen and Turner (1983) found that the natural abundance and ^{15}N isotope dilution methods provided similar estimates of $\underline{P}$ for subterranean clover, whereas Edmeades and Goh (1979) obtained different estimates of $\underline{P}$ with these two methods for white clover. Edmeades and Goh (1979) concluded that the natural ^{15}N abundance method should not be used until further work has established the validity of the assumptions

inherent in the method, especially with regard to N isotope fractionation effects.

Some of the assumptions implicit in the ^{15}N isotope dilution method, especially those concerning the reference plant, are also important for the natural ^{15}N abundance method. For example, it is assumed that all of the N in the reference plant is derived from the soil, i.e. (i) that there is no N_2 fixation directly associated with the reference plant (see section 2.4.3.4) and (ii) that no direct transfer of fixed N_2 from the legume to the reference plant takes place during the measurement period (see section 2.4.3.5). Due to the small differences in isotopic composition between atmospheric and soil N sources, when applying the natural ^{15}N abundance method it is also assumed that no isotopic fractionation occurs during sample preparation and ^{15}N analysis, or during the growth and N assimilation by the legume and reference plant.

2.4.4.1 Isotopic fractionation during N_2 fixation

Estimates of the isotopic fractionation which occurs during N_2 fixation by bacteria, crop legumes and pasture legumes are given in Table 2.6. Most of the estimates indicate that discrimination against the heavier ^{15}N isotope generally occurs (i.e. a negative $\delta^{15}N$ value is obtained).

To obtain an accurate estimate of $\underline{P}$ it is necessary to establish an isotopic fractionation factor for N_2 fixation (B) and to account for this by changing equation 21 to:

$$\underline{P} = \frac{\delta^{15}N_{\text{available soil N}} - \delta^{15}N_{\text{legume}}}{\delta^{15}N_{\text{available soil N}} - B} \qquad (22)$$

Table 2.6. Isotopic fractionation during N_2 fixation by bacteria, crop legumes and pasture legumes.

Species	Variety	B[1]	S.E.	Source
Bacteria				
Azotobacter agile		-1.5		Hoering and Ford 1960
chroococcum		-0.7		
indicum		+3.7		
vinelandi		-2.3		
vinelandi		-3.9		Delwiche and Steyn 1970
Crop legumes				
Glycine max L. Merrill	Amsoy	-1.2	0.2	
	Wells	-1.3	0.1	Amarger et al. 1979
	Chippewa	-1.5	0.4	
	Hodgson	-1.6	0.2	
	Harosoy	+0.98	0.18	Kohl and Shearer 1980
Lupinus luteus L.	Sulfa	-0.9	0.1	Armager et al. 1977
	Sulfa	-0.77		
Phaseolus vulgaris L.	Contender	-1.97		
	Hodgson	-1.83		Mariotti et al. 1980
Vicia faba L.	Ascott	-0.63		
Pisum sativum L.	Rondo	-1.0		
Pasture legumes				
Medicago sativa L.	Mireille	-0.92		Mariotti et al. 1980
Trifolium pratense L.	Alpilles	-0.88		
Trifolium pratense L.		+1.88	0.14	Kohl and Shearer 1980
Trifolium subterraneum L.	Mt. Barker	+2.58		Bergersen and Turner 1983

[1] $B = \delta^{15}N$ (with respect to atmospheric N_2) of the bacteria or legume grown solely on atmospheric N_2.

where B is the $\delta^{15}N$ of the legume grown solely on atmospheric N_2 (Amarger et al. 1979).

2.4.4.2 Isotopic fractionation during assimilation of soil nitrogen

As soil N is assimilated by both the legume and reference plant it is important that any isotopic fractionation which occurs during uptake of soil N should be the same for both plants if accurate estimates of P are to be obtained (Kohl and Shearer 1980).

Mariotti et al. (1980) examined 38 non-legumes and four uninoculated legumes for isotopic fractionation during assimilation of nitrate from a flowing solution. Uninoculated lucerne was the only plant to show significant fractionation during N uptake, having a $\delta^{15}N$ value of -1.0‰ with respect to the nitrate. In contrast, Kohl and Shearer (1980) used a hydroponic culture to grow non-nodulated soybeans, marigolds and ryegrass and measured isotopic fractionation with $\delta^{15}N$ values of -4.9, -4.5 and -4.7‰ respectively, relative to nitrate. However, they noted that these values contained errors due to the inability to maintain an infinite N source. Therefore fractionation associated with depletion of a finite source (Mariotti 1982) may have occurred. It should be noted that in the case of marigolds and ryegrass, the values for isotopic fractionation decreased with time to -3.1 and -1.9‰ respectively after 65 days. Mariotti et al. (1980) also measured changes in isotopic fractionation with time during nitrate uptake by millet (Pennisetum americanum L.), being large during early growth and becoming nil after 92 days growth (Fig. 2.1).

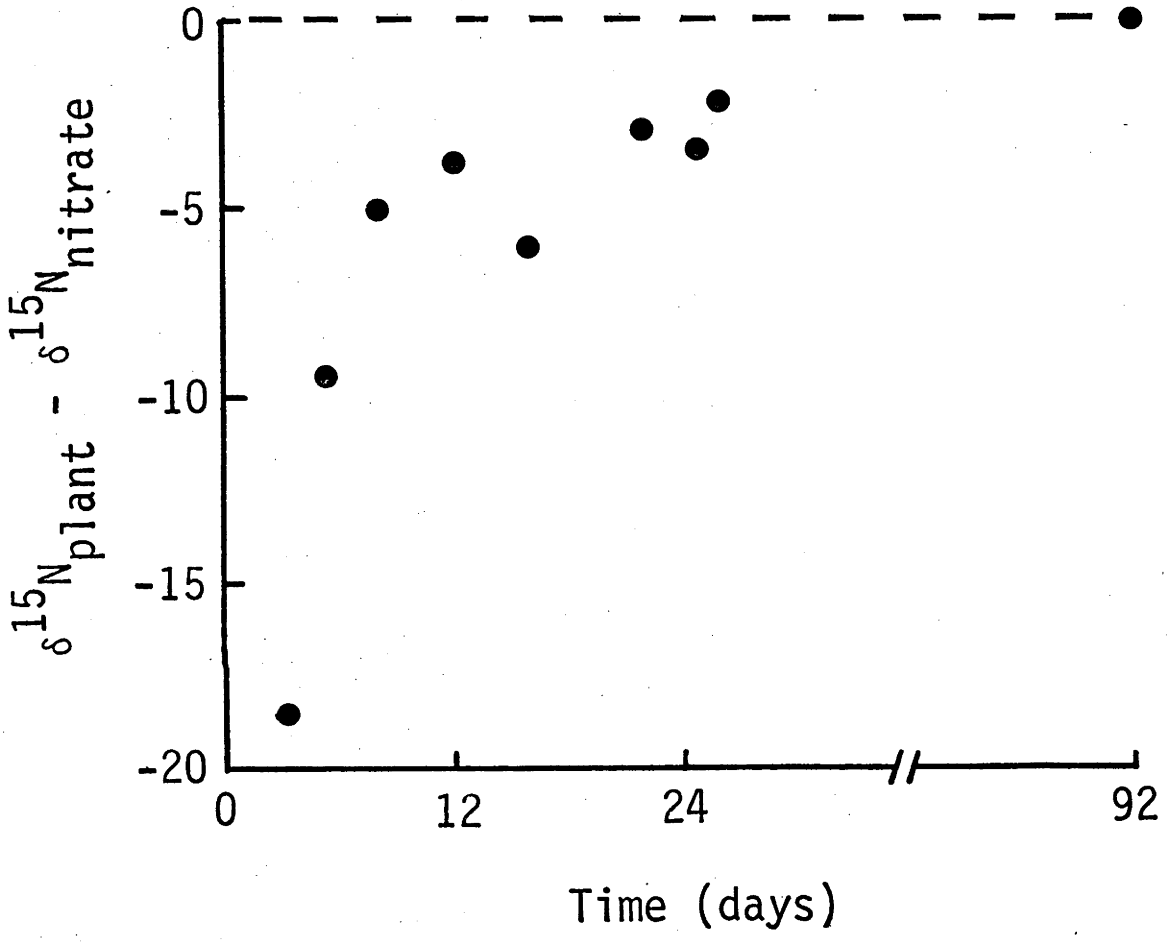


Figure 2.1. Isotopic fractionation during assimilation of nitrate by millet during growth (Mariotti et al. 1980).

Mariotti et al. (1982) examined the kinetics of nitrate uptake and utilization by millet and found that the level of isotopic fractionation depended on the relative concentrations of nitrate supplied to the plant, nitrate reductase within the plant and internal reductant. They observed that when nitrate reductase was rate-limiting (e.g. during early growth) isotopic fractionation was large, but when nitrate concentrations were low (as is typical for field conditions), nitrate uptake would be the rate-limiting step and therefore fractionation would be minimal. In the field, Karamanos and Rennie (1980) found no significant fractionation during assimilation of soil N by oilseed rape using a N balance study.

2.4.4.3 Isotopic fractionation associated with nitrogen translocation in plants

In a field study of N_2 fixation, it is more convenient to sample only a portion of the plant (e.g. shoots) for ^{15}N analysis, rather than the whole plant. Therefore, it is of interest to examine the ^{15}N abundance of N in different plant parts. The $\delta^{15}N$ values given in sections 2.4.4.1 and 2.4.4.2 are for the whole plants and may be different from those for the individual plant parts.

Nodules of soybeans, cowpeas and beans are generally enriched in ^{15}N relative to other plant parts, with $\delta^{15}N$ values of +4.4 to +10.4‰ relative to other plant parts, having been observed (Shearer et al. 1982). However, this enrichment has not been found with many other legumes and Shearer et al. (1982) hypothesized that it only occurred in ureide-transporting plants. Anomalies to this hypothesis have since been found (Steele et al. 1983; Turner and Bergersen 1983). Steele et al. (1983) found that the strain of

rhizobium affected the degree of ^{15}N enrichment of nodules with soybean and white clover. However, because nodules only make up a small part of the whole plant this will only have a small effect on the ^{15}N abundance of the total plant N.

Apart from the nodules, Shearer et al. (1980) found little variation in $\delta^{15}N$ between plant parts of soybean, whether nodulated or non-nodulated, with the deviation from the whole plant generally being 1‰ or less. However, this still had a significant effect on the estimate of P. For example, the respective $\delta^{15}N$ values for whole plants and shoots of nodulated soybean were +3.4 and +2.4‰ (with respect to atmospheric N_2), while those for non-nodulated soybean were +7.0 and +7.4‰, giving P values of 51.4 and 67.7%. In contrast, Bergersen and Turner (1983) found little effect on P of a consistently higher ^{15}N abundance in roots than in shoots of subterranean clover and ryegrass in the field.

2.5 Concluding remarks

Before plant breeders can develop legumes with increased levels of N_2 fixation and agronomists can establish the importance of management practices on N_2 fixation, it is essential to establish a reliable method for measuring N_2 fixation by legumes in the field. Many indirect and direct methods have been used to estimate N_2 fixation by legumes in the field and each of these have various advantages and disadvantages (see Table 2.7). Although the indirect acetylene reduction method and the more direct N difference method have been widely used, in general they do not provide sufficiently accurate estimates of N_2 fixation to enable researchers to study the effects of management practices on N_2 fixation by legumes in the field

Table 2.7. Summary of the advantages and disadvantages of the methods for estimating N_2 fixation by legumes in the field.

Advantages	Disadvantages
1. Ureide method	
: inexpensive	: indirect (must establish relationship with N_2 fixation, which may vary)
: relatively simple	: limited to a small group of legumes
2. Acetylene reduction method	
: inexpensive	: indirect (must establish conversion factor for N_2 fixation, which may vary)
: simple	: C_2H_2 may inhibit nitrogenase activity
: rapid	: difficult to maintain environmental conditions pertaining to the field
: very sensitive	: inaccurate at low levels of nitrogenase activity in field soils
	: must integrate many short-term measurements
3. Response by non-legumes	
: inexpensive	: indirect (only measures transfer of fixed N_2 to non-legumes)
: relatively simple	: difficult to get suitable control (crop legumes only)
	: plant-availability of fertilizer N and decomposing legume N may differ (N fertilizer equivalence method only)
4. Total nitrogen accumulation	
: direct	: includes legume N derived from soil
: inexpensive	: may need long term study (where Δ soil N is measured)
: simple (excluding N balance method)	
5. Difference methods	
: direct	: requires suitable non-N_2 - fixing reference plant
: relatively simple	: legume and reference plant must absorb the same amount of soil N
: correct for soil-derived N	

Table 2.7 (contd.)

Advantages	Disadvantage
6. $^{15}N_2$ method	
: direct	: very expensive
: accurate	: difficult to establish and maintain
	: must integrate many short-term measurements
	: difficult to maintain environmental conditions pertaining to the field
7. A-value & ^{15}N isotope dilution methods	
: direct	: expensive
: potentially accurate	: requires addition of ^{15}N-labelled compound
: can get yield-independent estimate of % N fixed (^{15}N isotope dilution method only)	: requires suitable non-N_2-fixing reference plant
	: legume and reference plant must absorb the same relative amounts of N from the soil soil and added N
8. Natural ^{15}N abundance method	
: direct	: relatively expensive
: no ^{15}N addition required	: requires suitable non-N_2-fixing reference plant
: can get yield-independent estimate of % N fixed	: insensitive if $\delta^{15}N_{soil}$ nears $\delta^{15}N_{air}$
	: field variability may be large
	: may have to allow for isotopic fractionation during assimilation of N_2 or soil N

(Phillips and Bennett 1978). The ^{15}N isotope dilution and natural ^{15}N abundance methods appear to be the most promising for studies of this kind.

There has been an increase in the use of these two ^{15}N techniques in recent years, particularly with crop legumes such as soybeans, but there are still relatively few studies involving pasture legumes.

The assumptions inherent in these ^{15}N methods have been recognized but there have been no detailed evaluations of their importance in the estimation of N_2 fixation. The study reported in this thesis examines each of the assumptions of the ^{15}N isotope dilution and natural ^{15}N abundance methods and determines their relative importance in estimating N_2 fixation by two pasture legumes.

CHAPTER 3

MATERIALS AND METHODS

3.1 Introduction

In this chapter, the materials and methods of general use are described. Within each of the subsequent chapters, details specific to the experiments described in those chapters are given.

3.2 Soils and sites

A range of soils were used in the study on variability in the natural abundance of ^{15}N in soil N (Chapter 4) and their characteristics are given in Table 3.1. Parent rock from soils 2,3 and 4 (i.e. granite, sediment and basalt) was also collected, by removing samples from outcrops within the areas in which the soils were sampled.

In two of the studies on the relative uptake of added N and indigenous soil N by plants (Chapter 10) it was necessary to use a soil that had previously been enriched in ^{15}N. Therefore, a site was chosen at the CSIRO Ginninderra Experimental Station, Australian Capital Territory, that contained pasture plots which had received 1.05 kg N ha^{-1} as $NaNO_3$ (95 atoms % ^{15}N), three years previously. The soil was classified as a yellow podzolic soil (Gn 3.85; Northcote 1971) or a Andaqueptic Ochraqualf (Sleeman 1979). It was located on colluvium/alluvium and derived from porphyritic dacite (Sleeman 1979). In pot experiment 4 (section 10.3.2) the 0-100 mm layer of soil was used whereas in the soil profile experiment (section 10.3.3), soil from the 0-100, 100-200 and 200-400 mm layers was used.

Table 3.1. Description of soils.

Soil	1	2	3	4	5
Location	Canberra	Pejar Dam Catchment			Canberra
Soil description	solodic with overburden	yellow duplex	yellow duplex	red duplex	yellow podzolic
Northcote key[1]	Dy2.42/Uc5.23	Dy3.41	Dy3.41	Dr4.12	Gn 3.85
Parent material	granite	granite	sedimentary	basalt	granite
Years under subterranean clover	10	0,25,55	0,25,55	0,25,55	15

[1]Northcote (1971).

Most experimental work involved the use of a yellow podzolic soil, with the same features as the soil described above, located at the Ginninderra Experimental Station. This experimental site was chosen for its horizontal and vertical uniformity in soil texture. The 0-100 mm soil layer had a pH of 6.04 and total N concentration of 0.138%. Soil was collected from the edges of this site for pot experiments 1 and 3, and the main area of the site was prepared for field studies on N_2 fixation with four legume/grass associations.

3.2.1 Site preparation for field studies

The site had previously been used for an experiment on the productivity of various grass species and had been subject to light, irregular grazing by sheep. In October 1981, it was sprayed with glyphosate to kill existing vegetation and then cultivated, fertilized with molybdic superphosphate (350 kg ha^{-1}) and sown to oats (Avena sativa L. Blackbutt) to reduce the level of plant-available soil N prior to the establishment of the experiment. Oat herbage was removed with a forage harvester in January and the site was sprayed with paraquat in February 1982, to kill any regrowth. Several weeks later the site was irrigated and then given a shallow (0-100 mm) cultivation. Superphosphate (250 kg ha^{-1}) and a solution of sodium tetraborate (5 kg ha^{-1}) and sodium molybdate (240 g ha^{-1}) were then applied to the site and it was recultivated.

On 6 April 1982, plots (10 m x 1.5 m) were marked out in a randomised block design with six replicates and sown to five species combinations. The species used were subterranean clover (Trifolium subterraneum L. cv. Woogenellup), lucerne (Medicago sativa L. cv. Siriver), annual ryegrass (Lolium rigidum Gavdin. cv. Wimmera) and

phalaris (Phalaris aquatica L. cv. Sirosa) sown at 10.9, 9.1, 6.2 and 10.3 kg ha^{-1} respectively in the four legume/grass combinations. Ryegrass-only plots were also prepared.

3.3 Analytical procedures

3.3.1 Measurement of soil pH

Soil (10 g) was shaken with 50 ml of CO_2-free water for one hour and the pH of the suspension was determined with a glass electrode/calomel electrode assembly and millivolt meter.

3.3.2 Nitrogen analysis

All extracts and samples of plant and soil to be analysed for N were converted to ammonium-N (NH_4^+-N) and subsequently analysed for ^{15}N. Therefore, care was taken to avoid contamination or isotopic fractionation during N analysis, as outlined in the following sections.

3.3.2.1 Total nitrogen in plant material

Plant samples were oven-dried at 70°C for 48 hours, weighed and ground to a very-fine powder in a N.V. Tema puck mill (Siebtechnik GmbH, Mülheim, Germany). They were then stored in stoppered glass vials and redried prior to analysis. Oven-dry samples containing about 1 mg N were accurately weighed and placed in Pyrex test-tubes with 4 ml of digestion mixture and heated at 310°C in a block digester.

The digestion mixture was prepared by heating 1 l H_2SO_4, 100 g K_2SO_4 (to elevate the boiling point of the mixture) and 1 g powdered Se (to act as a catalyst) at 250-300°C for 3-4 hours until clear (Bergersen 1980). After cooling, 25 g salicylic acid was added to ensure complete conversion of nitrate (NO_3^-) in the plant material to NH_4^+ (Bremner 1965a).

The salicylic acid modification of the conventional Kjeldahl method was selected after comparing it with the reduced iron modification of Goh (1972). Total N in 15 mg of plant material, with and without the addition of 140 µg NO_3^--N, was determined by the conventional Kjeldahl method or as modified with salicylic acid or reduced iron. Recovery data presented in Table 3.2, show that both modified methods increased recovery of NO_3^--N and that the total N contents, as determined by these two methods, were not significantly different. The salicylic acid modification was chosen for subsequent analyses because results obtained with this method were less variable than those obtained with the reduced iron modification (Table 3.2).

It has been shown (McKenzie and Wallace 1954; Bremner 1960) that temperature is the most important factor controlling digestion. Therefore, to ensure complete conversion of organic N to NH_4^+-N and to avoid loss of NH_3, the temperature of the digestion mixture was maintained at 310°C and the tops of the test-tubes were covered with glass funnels to prevent loss of acid. Digestion was continued until 1½ hours after the mixture had cleared. The digested samples were then cooled and sealed to avoid absorption of NH_3 from the atmosphere.

Two reagent blanks (i.e. with no plant material) were digested with each set of 48 samples so that allowance could be made for any N present in the reagents (Bergersen 1980). Within an experiment, two replicates of one sample were also repeatedly analysed with each set of 48 samples to check for possible variation in isotope composition of N with time.

Steam-distillation was used to recover NH_4^+ from the digestion mixture. This involved transferring the digest quantitatively to a

Table 3.2. Effect of method of analysis on the recovery of NO_3^--N in the Kjeldahl method for total N determination in plant material. Each value is the mean of four replicates and values in brackets are standard errors.

	Total nitrogen (µg N)		% recovery of added NO_3^--N
	no added N	+ 140 µg NO_3^--N	
Conventional Kjeldahl	815 (6)	883 (15)	49
Salicylic acid	810 (4)	937 (2)	91
Reduced iron	813 (13)	945 (7)	94
S.E.D.	11	13	12

steam distillation flask, adding 20 ml 10 N NaOH, connecting the flask to the glass distillation apparatus and collecting the distillate in 10 ml 1% boric acid.

It has been suggested that a stainless steel distillation unit should be used to prevent adsorption of NH_4^+ to the walls of the still (Buresh et al. 1982). To test this suggestion, a plant digest, at natural abundance of ^{15}N, was analysed for NH_4^+-N in an all-glass unit or in a stainless steel unit and varying volumes of distillate were collected. The results (Fig. 3.2) show that there was no advantage in using stainless steel and that 40 ml of distillate was sufficient to avoid isotopic fractionation of N. However, to ensure complete recovery of N and to avoid contamination between samples, 80 ml of distillate was collected for all samples, as suggested by Turner and Bergersen (1981).

Whenever samples with greatly different ^{15}N enrichment were to be analysed, samples of unenriched $(NH_4)_2SO_4$ (analytical reagent, B.D.H. chemicals, Australia) were first distilled to desorb any previously adsorbed NH_4^+-N and thus prevent cross-contamination (Reeder et al. 1980). Problems with cross-contamination were only encountered when highly ^{15}N-enriched samples (e.g. fertilizer N at 10-90 atoms % ^{15}N) were distilled immediately prior to samples of low enrichment or at natural abundance of ^{15}N.

To check that there was no isotopic fractionation during the digestion and distillation procedures, samples of unenriched $(NH_4)_2SO_4$ from a bulk solution were analysed for ^{15}N and compared with samples that had been digested and distilled or distilled only. The results (Table 3.3) show that there was no isotopic fractionation of N during digestion or distillation.

Figure 3.1. Effect of still material (stainless steel ●—● ; glass ○—○) and volume of distillate collected on a) the amount of nitrogen collected and b) its isotopic composition. Each value is the mean of three replicates and bars represent average S.E.D.'s.

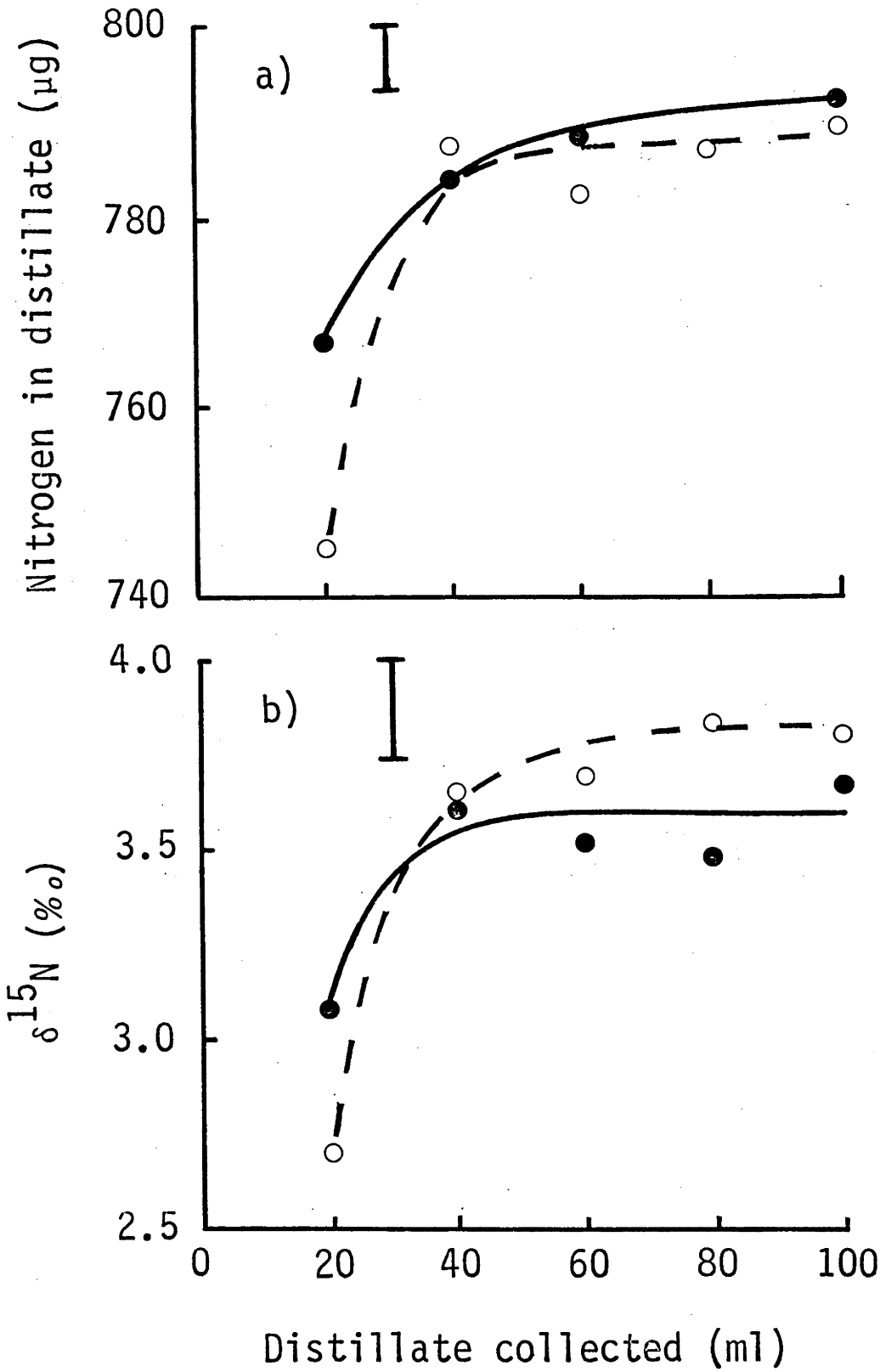

800
780
760
740
a)
Nitrogen in distillate (µg)
4.0
3.5
3.0
2.5
b)
$\delta^{15}N$ (‰)
0
20
40
60
80
100
Distillate collected (ml)

Table 3.3. Effect of digestion and/or distillation of a sample of ammonium sulphate solution on the $\delta^{15}N$ (with respect to ammonium sulphate reference). Each value is the mean of four replicates.

	sample	distilled sample	digested & distilled sample	S.E.D.
$\delta^{15}N(‰)$	1.257	1.292	1.105	0.155

The amount of NH_4^+-N in the distillate was determined by potentiometric titration with 0.01 N HCl. One drop of 1% H_2SO_4 was then added to reduce the pH of the titrated distillate to about 4.0, to ensure no NH_3 loss occurred during concentration of the solution to about 2 ml by boiling. The solution then contained about 1 mg N ml^{-1}. The flask containing the concentrated sample was sealed with a rubber stopper, to prevent absorption of atmospheric NH_3, until analysed for ^{15}N.

3.3.2.2 Total nitrogen in soil

Air-dry soil samples were ground to pass through a 0.125 mm aperature sieve and analysed for total N by a salicylic acid modification of macro-Kjeldahl method (Bremner 1965a).

Soil samples containing about 10 mg N were accurately weighed and placed in 450 ml Kjeldahl flasks with 40 ml of salicylic acid: H_2SO_4 (25 g : 1 l) mixture, stoppered and left overnight. Five g sodium thiosulphate (< 20 mesh) was then added and the mixture cautiously heated until frothing ceased. Flasks were then cooled and 10 g K_2SO_4, 1 g $CuSO_4.5H_20$, 0.1 g Se and 10 ml concentrated H_2SO_4 were added. The mixture was then heated at 310°C until the digest turned a pale yellow colour; the digestion was then continued for a further five hours. The digested samples were cooled, made up to volume (400 ml) with distilled water and stoppered. Forty ml aliquots were steam-distilled with 25 ml of 10 N NaOH and prepared for isotopic analysis as for the plant samples.

This method for total N determination was selected after comparison with a reduced iron modification of the Kjeldahl method (Goh 1972). Recovery of NO_3^--N and sensitivity were found to be superior with the salicylic acid modification (Table 3.4).

Table 3.4. Effect of method of analysis on the recovery of NO_3^--N added to 7g yellow podzolic soil. Each value is the mean of four replicates and values in brackets are standard errors.

	Total N (µg)		% recovery of
	no added N	+ 125 µg NO_3^--N	added NO_3^--N
Salicylic acid	898 (2)	1,022 (4)	99
Reduced iron	889 (2)	973 (29)	67
S.E.D.	3	29	23

3.3.2.3 Total nitrogen in rocks

Rock samples were mechanically crushed and ground to a fine powder in a N.V. Tema puck mill.

Samples containing about 10 mg N were weighed and digested in 450 ml Kjeldahl flasks with 10 g K_2SO_4, 1 g $CuSO_4.5H_20$, 0.1 g Se and 40 ml concentrated H_2SO_4 at 310°C for 12 hours after the mixture had turned yellow. Forty ml aliquots were then steam distilled with 25 ml 10 N NaOH and prepared for isotopic analysis as for the plant samples.

3.3.2.4 Inorganic nitrogen in soil

Inorganic N in soil samples was extracted within six hours of collection, to minimise mineralization of organic N. When the inorganic N concentration was very low (e.g. < 10 µg N g^{-1} oven-dry soil), soils were extracted by shaking 150 g soil with 450 ml 0.3 N KCl for one hour and centrifuging. The supernatant was then passed through Whatman No.42 filter paper and evaporated to 50 ml in preparation for distillation (Black and Waring 1977).

When soils had a low inorganic N concentration but were enriched in ^{15}N (e.g. following addition of ^{15}N enriched fertilizer), smaller soil samples were used and a known amount of unenriched $(NH_4)_2SO_4$ of known isotopic composition was added to the extracted inorganic N as a carrier prior to isotopic analysis. These ^{15}N-enriched soils were extracted by shaking 50 g soil with 150 ml 2 N KCl for one hour and centrifuging. The supernatant was then filtered through Whatman No.42 paper prior to distillation. This extraction procedure was also used when soil samples exceeded 10 µg inorganic N g^{-1} (e.g. soils that had been incubated).

To avoid contamination by NH_4^+-N present in the filter papers (Buresh et al. 1982), these were preleached with 50 ml 2 N KCl before use.

The steam-distillation procedure of Bremner and Keeney (1965) was used to recover NH_4^+-N and NO_2^--N plus NO_3^--N from the soil extracts. Ammonium-N was distilled from the soil extracts using MgO (6 ml 12% w/v suspension in distilled water), and the distillate was collected in 10 ml of 1% boric acid as for total N analysis (see section 3.2.2.1.). After the NH_4^+-N had been completely removed, 2 g Devarda's alloy (45% Al, 50% Cu, 5% Zn; < 0.125 mm particle size) was added to the alkaline suspension to reduce NO_2^- and NO_3^- to NH_4^+, and the distillation was continued until a further 80 ml of distillate had been collected. For the determination of total inorganic N (NH_4^+ + NO_2^- + NO_3^-), the MgO and Devarda's alloy were both added at the beginning of the distillation. Preparation of samples for isotopic analysis was as described for plant material.

3.3.2.5 Chemical fractionation of soil nitrogen

Soil N was fractionated into fulvic acid, humic acid and humin (Kononova 1966). To achieve this a mixture of soil and 0.1 N NaOH (1:10) was shaken for one hour at 20°C. The mixture was then centrifuged at 1250 g for 30 minutes to separate the residue (humin) from the supernatant, which was decanted through a pre-leached Whatman No. 42 filter paper to remove floating debris. The filtrate was adjusted to pH 1.0 with H_2SO_4, left to stand for three hours and centrifuged at 1250 g for 20 minutes to separate the precipitate (humic acid) and supernatant (fulvic acid). The supernatant was decanted into a beaker and reduced in volume by boiling. All three fractions were then digested (conventional Kjeldahl method), distilled and prepared for ^{15}N analysis as for total soil N.

3.3.2.6 Physical fractionation of soil nitrogen

Soils were separated into different particle size fractions by dispersion in water, sieving and centrifugation. A soil:water mixture (1:10) was dispersed in water with the aid of an ultrasonic vibrator (Watson and Parsons 1974) and passed through a 53 μm sieve to separate the sand-sized fraction. The filtrate was then centrifuged at 61 g (calculated at top of the sediment) for 6 minutes to separate the silt-sized (2-53 μm) and clay-sized (< 2 μm) fractions (Genrich and Bremner 1974). The three fractions were acidified with H_2SO_4, reduced in volume by boiling, digested (conventional Kjeldahl method), distilled and prepared for ^{15}N analysis as for total soil N.

3.3.3 ^{15}N analysis

The concentrated NH_4^+-N (0.5-1.5 mg N ml^{-1}) samples prepared following digestion, distillation and titration, were converted to N_2 for mass spectrometric ^{15}N analysis by a modified Rittenberg technique, as described by Bergersen (1980). Care was taken at all stages to ensure complete conversion of sample N to NH_4^+-N and then to N_2 so that there was no isotopic fractionation.

A Rittenberg vessel has two limbs joined in an inverted Y-shape; one containing the acidified NH_4^+ sample and the other containing 2 ml of alkaline NaOBr. The alkaline NaOBr was prepared by dissolving 8 ml of bromine in 70 ml of distilled water and chilling in ice. Forty ml of NaOH solution (prepared by mixing 29 g NaOH with 40 ml distilled water) was then added to the bromine solution. Alkaline NaOBr prepared in this way lasted 2-4 days and did not result in problems due to O_2 evolution (i.e. $2NaOBr \rightarrow 2NaBr + 0_2$) as found by others (e.g. Bremner 1965b).

Key steps in the subsequent procedure were thorough degassing of sample and reagent solutions under vacuum and drying of the N_2 sample before entry into the mass spectrometer, because O_2 and water vapour can cause instability of ionisation in the mass spectrometer and can lead to erroneous changes in the mass 28 and 29 signals (Bremner et al. 1966; Martin and Ross 1968).

The Rittenberg vessel was attached to a vacuum line and evacuated to remove air, including that dissolved in the reagent or sample. Care was taken during evacuation to avoid the reagent splashing over into the sample. This would result in isotopic fractionation due to some loss of N from the sample. The N remaining would have a higher ^{15}N concentration than that in the original sample. After about 15 minutes evacuation the stopcock on the vessel was closed so that the vessel remained under vacuum and the reagent and sample was mixed together, resulting in the following reaction (Bremner 1965b):

$$3\ NaOBr + 2NH_3 \rightarrow N_2 + 3\ NaBr + 3H_20.$$

For the reaction to go to completion the mixture must be alkaline. When there was too large an excess of acid in the NH_4^+ sample, this reaction was incomplete and a low ^{15}N concentration resulted.

The Rittenberg tube was then attached to the inlet of the isotope ratio mass spectrometer (Micromass 903E; VG Isogas, Middlewich, Cheshire, U.K.) equipped with a double inlet system (for sample and reference) and triple collector plates (for mass 28, 29 and 30). Before admission of the sample N_2 into the mass spectrometer, the limbs of the Rittenberg vessel were immersed in a dry-ice/ethanol mixture (temperature approximately - 80°C) to freeze out any

contaminating higher oxides of N, water and bromine. Bremner (1965b) recommended the use of liquid N_2 in this step. However, it has been found (Bergersen 1980) that liquid N_2 reduces the amount of N_2 gas, presumably by adsorption on ice crystals (e.g. Martin and Ross 1968) and this can cause isotopic fractionation.

While the reacted sample was being frozen, the connection between the Rittenberg vessel and the mass spectrometer was pumped out with the inlet vacuum system of the mass spectrometer. The line to the vacuum pumps was then closed and the sample N_2 was expanded into the sample reservoir. The line to the Rittenberg vessel was then closed. Any air leakage or water vapour present generally causes the mass 28 signal to increase or decrease, respectively. When this occurred, the sample was immediately pumped out and another sample was prepared and introduced. Another check for leakage was to scan the sample for mass 32 (oxygen) and mass 40 (argon) peaks. The presence of oxygen alone cannot be used as a measure of atmospheric contamination because decomposition of NaOBr can also lead to the production of oxygen (as discussed above).

A solution of $(NH_4)_2SO_4$ (analytical reagent, B.D.H. chemicals, Sydney) containing 1 mg N ml^{-1} was used as a reference. This was prepared in bulk, because the isotopic composition of individual crystals can differ (Turner and Bergersen 1981), and frozen in 15 ml quantities until required. The $(NH_4)_2SO_4$ reference was converted to N_2, as described above, and expanded into the reference reservoir of the mass spectrometer.

The mass 28 signals of the reference and sample were then equalised by adjustment of the volume of reference and sample reservoirs of the mass spectrometer. Isotopic determination involved

alternating the input of sample and reference to the analyser of the mass spectrometer (via an automatic changeover valve) six times to obtain a measurement of the isotopic composition of the sample relative to the reference. Bremner (1965b), Hauck and Bremner (1976), Bergersen (1980) and Buresh et al. (1982) provide details of the functioning of a mass spectrometer.

To prevent contamination of one sample with another by hold-up of N in the mass spectrometer inlet system, a sufficiently long pumping period was used between samples. Also, batches of samples with a similar isotopic composition were analysed consecutively. When samples with a range in isotopic composition were being analysed, they were always run in order from lowest to highest ^{15}N abundance during both distillation and isotopic analysis.

Contamination can also occur due to mixing of sample and reference gases in the change-over valve due to seat leakage. This was found to be insignificant, except when changing from an ^{15}N-enriched to a natural abundance sample; this results in an underestimation of the ^{15}N abundance of the latter. In this case, a longer pumping-out period and a new reference gas were used.

The N_2 to be analysed occurs as mass 28 ($^{14}N^{14}N$), 29 ($^{14}N^{15}N$) and 30 ($^{15}N^{15}N$) and the relative number of these atoms occur in the proportions $p^2 : 2pq : q^2$, where p and q are the number of ^{14}N and ^{15}N atoms respectively. The ratio (r) of the mass 29 and 28 signals is measured and can be described by

$$r = \text{mass } 29/\text{mass } 28 = 2pq/p^2 = 2q/p. \qquad (23)$$

The proportion of ^{15}N atoms of the total ($^{14}N + {}^{15}N$) is commonly given as

$$\text{atoms \% } {}^{15}N = 100q/(p + q). \quad (24)$$

By rearranging, this can be written as

$$\text{atoms \% } {}^{15}N = 100r/(2 + r). \quad (25)$$

At the level of natural abundance of ^{15}N, the following expression was used:

$$\delta^{15}N = 1000\ (r_{sample} - r_{reference})/r_{reference} \quad (26)$$

However $\delta^{15}N$ values are commonly given with respect to atmospheric N_2 and therefore the $\delta^{15}N$ of the $(NH_4)_2SO_4$ reference solution was compared with that of oxygen-free atmospheric N_2, which was prepared as follows (G.L. Turner, unpublished): 1 ml 10 N NaOH and 0.15 mg $Na_2S_2O_4$ were placed in either limb of a Rittenberg vessel, evacuated on a vacuum line, adjusted to 0.1 atm air, mixed and left to stand for one hour before ^{15}N analysis.

The relationship between $\delta^{15}N$ and atoms % ^{15}N is obtained by rearranging equation 26 to give

$$r_{sample} = r_{standard}\ [1 + (\delta^{15}N_{sample}/1000)] \quad . \quad (27)$$

Now $r_{standard}$ (where the standard is O_2-free atmospheric N_2) can be calculated from equation 25 for atmospheric N_2 by making atoms % ^{15}N = 0.3663 (Junk and Svec 1958) and rearranging. Then

$$r_{standard} = \frac{2 \times 0.3663}{100 - 0.3663} = 0.0073529. \quad (28)$$

Thus, the relationship between $\delta^{15}N$ and atoms % ^{15}N is obtained by substituting from equations 27 and 28 into equation 25, for the sample to give:

$$\text{atoms \% } {}^{15}N_{sample} = \frac{100 \times 0.0073529\ [1 + (\delta^{15}N_{sample}/1000)]}{2 + 0.0073529\ [1 + (\delta^{15}N_{sample}/1000)]} \quad . \quad (29)$$

The reproducibility of ^{15}N analysis is illustrated by the variation obtained in analysing replicates of a single sample (Table 3.5). This variability is due, at least in part, to the difficulty in obtaining a representative subsample and to errors in sample preparation. Some indication of the potential error associated with the mass spectrometer was obtained when a sample of N_2 was equilibrated between the sample and reference reservoirs of the mass spectrometer and analysed; $\delta^{15}N$ values of up to $\pm 0.03‰$ were recorded.

The day-to-day variability in ^{15}N analysis (Table 3.6) was found to be little more than that for a sample analysed within one day (compare Tables 3.5 and 3.6).

3.3.4 Acetylene reduction assays

The effect of low rates of N addition on N_2 fixation by subterranean clover (Chapter 7) was examined by regular non-destructive acetylene reduction assays conducted in specially constructed assay vessels (Plate 3.1). The vessels were made gas-tight by sealing the draining hole with a rubber stopper and connecting the perspex top to the vessel with vacuum grease and adhesive tape (Plate 3.1). During the assays, C_2H_2 and propylene (C_3H_6) were injected into the vessel through the subaseal in the side port of the vessel and gas samples were collected through the subaseal in the perspex top.

In the field, two acetylene reduction methods were used to examine N_2 fixation associated with ryegrass (Chapter 8). The closed system involved the collection of fifteen soil cores (30 mm diameter x 800 mm long). These were placed in an assay vessel, consisting of a 2 l plastic jar with a screw-on plastic lid. The lid had a hole into which a subaseal was inserted. Several layers of thin plastic

Table 3.5. Reproducibility of analyses for $\delta^{15}N$ [in ‰ with respect to an $(NH_4)_2SO_4$ standard] in plant and soil nitrogen.

	Plant	Soil	
	Total N	Total N	Inorganic N
	1.886	6.153	1.354
	2.026	5.758	0.991
	2.102	6.178	1.838
	2.142	5.453	1.030
	1.900	5.778	1.769
	2.255	5.597	0.606
Mean	2.052	5.820	1.265
S.E.	0.059	0.120	0.196

Table 3.6. Variation in the $\delta^{15}N$ (in ‰ with respect to an $(NH_4)_2SO_4$ standard) of total plant nitrogen when analysed on different dates.

	2.709
	2.777
	3.054
	2.576
	3.213
	3.403
Mean	2.955
S.E.	0.131

Plate 3.1. Acetylene reduction system used to study the effect of inorganic nitrogen on nitrogenase activity; from left to right, an assay vessel containing subterranean clover located on a plastic draining saucer to collect leachate, an assay vessel with perspex top attached for injection of acetylene and propylene (through subaseal in side port of the vessel) and collection of gas samples (through subaseal in the top), and a Vacutainer tube used for storage of gas samples.


9

('Clingwrap') were placed over the top of the jar before applying the lid, to try to maintain a gas-tight seal. In the in situ open system, twelve metal cylinders (124 mm diameter, 200 mm long; Roper 1984) were driven into the ground (three weeks before the assay date) so that 50 mm extended above the soil surface. Before commencing the assay, metal plates were bolted to the rubber-gasket-lined flanges at the top of the metal cylinders. Each metal cap contained a vent into which a subaseal was inserted to allow injection of C_2H_2 and C_3H_6, and for the removal of gas samples.

In all assays, air was withdrawn from the assay vessels and replaced with C_2H_2 at 0.1 bar in the closed systems and 0.15 bar in the open system to ensure saturation of the N_2-fixing sites (Hardy and Holsten 1977). At the same time a small measured amount of C_3H_6 was also injected into each assay vessel to serve as an internal standard for C_2H_4, due to its similar rate of diffusion (Knowles 1980). Acetylene and C_3H_6 were transported to the assay site in mechanical gas reservoirs as described by Turner and Gibson (1980).

With the closed systems, initial studies were made on the rates of C_2H_4 production with time to determine when gases were equilibrated and when the rate of C_2H_4 production began to decline, so that appropriate times of sampling could be determined. The gases had equilibrated by 5 to 20 minutes after injection and therefore the first gas samples were collected at 20 to 30 minutes after injection of C_2H_2 and C_3H_6.

Double-ended sampling needles and pre-evacuated Vacutainer (Becton, Dickinson and Co., Rutherford, New Jersey, U.S.A.) blood-sampling tubes (10 ml volume) were used for sample collection

(Turner and Gibson 1980). Samples were collected in duplicate in case of needle blockages and as a check on variability.

Ethylene, C_2H_2 and C_3H_6 were analysed gas chromatographically. A gas-sampling syringe was used to inject 200 µl of gas from the Vacutainer sampling tubes into the gas chromatograph. Care was taken to ensure that there was no leakage from the gas-sampling syringe or the gas chromatograph. This involved regular replacement of the tape on the plunger of the syringe and of the septum of the gas chromatograph. Reduced peak heights of C_2H_2 and C_2H_4 and longer retention times served as an indicator of septum leakage. The peak heights (and attenuations) were measured from the printout of a chart recorder connected to the gas chromatograph.

To estimate the rate of C_2H_4 production, a sample containing equimolar quantities of C_3H_8 and C_2H_4 was also analysed. The ratio of their respective peak heights (p'/e') was then used to calculate the rate of C_2H_4 production (mol cm^{-2} hr^{-1}) as described by Knowles (1980):

$$\text{mol } C_2H_4 \text{ cm}^{-2}\text{hr}^{-1} = \frac{M}{a(t_2-t_1)} \times \frac{p'}{e'} \times \left(\frac{h_{e2}}{h_{p2}} - \frac{h_{e1}}{h_{p1}}\right) \qquad (30)$$

where M is the amount (mol) of C_3H_8 injected into the assay vessel, a is the surface area (cm^2) of the sample, and h_{e1}/h_{p1} and h_{e2}/h_{p2} are the ratios of the C_3H_8 and C_2H_4 peak heights obtained for the gas samples taken at times t_1 and t_2 respectively.

3.4 Calculation of the proportion (P) of legume N_2 fixed and its error

The proportion (P) of legume N derived from atmospheric N_2, when grown in soils at natural ^{15}N abundance or ^{15}N enriched, was derived as follows:

$$\underline{P} = \frac{\delta^{15}N_{\text{reference plant}} - \delta^{15}N_{\text{legume}}}{\delta^{15}N_{\text{reference plant}} - B} \quad (31)$$

or

$$\underline{P} = \frac{\text{atoms \% } ^{15}N_{\text{reference plant}} - \text{atoms \% } ^{15}N_{\text{legume}}}{\text{atoms \% } ^{15}N_{\text{reference plant}} - B} \quad (32)$$

where B is the $\delta^{15}N$ (equation 31) or atoms % ^{15}N (equation 32) of legume N derived entirely from atmospheric N_2 (as determined in pot experiment 2, Chapter 5).

In calculating the $\underline{P}$ value between two harvest intervals (1 and 2), a yield-dependent expression was used to obtain X (the average ^{15}N concentration of plant-available soil N during the period of measurement), based on the $\delta^{15}N$ or atoms % ^{15}N (a) of N assimilated by the legume (L) or reference plant (R) between time t_1 and t_2, e.g.

$$X = a_{R(t_2-t_1)} = (a_{R2}N_{R2} - a_{R1}N_{R1})/(N_{R2}-N_{R1}) \quad (33)$$

where N refers to the N yield. The yield-dependent expression of $\underline{P}$ is then given by

$$\underline{P} = \{X-[(a_{L2}N_{L2} - a_{L1}N_{L1})/(N_{L2}-N_{L1})]\} \; / \; (X-B). \quad (34)$$

Then $\underline{P}$ value is calculated from three independent parameters and therefore a series expansion to the first order (Davies and Goldsmith 1972) was used to estimate the variance associated with $\underline{P}$ as follows:

$$\text{var}(\underline{P}) \simeq \left(\frac{\partial L}{\partial \underline{P}}\right)^2 .\text{var}(L) + \left(\frac{\partial R}{\partial \underline{P}}\right)^2 .\text{var}(R) + \left(\frac{\partial B}{\partial \underline{P}}\right)^2 .\text{var}(B) \quad (35)$$

where L and R are the ^{15}N concentrations of the legume and reference plant and var is the variance.

Now,

$$\partial L/\partial \underline{P} = -1/(R-B) \quad ,$$

$$\partial R/\partial \underline{P} = (L-B)/(R-B)^2 \quad , \text{ and}$$

$$\partial B/\partial \underline{P} = -(R-L)/(R-B)^2 \quad .$$

Therefore, equation 35 can be rewritten as:

$$\text{var}(\underline{P}) = \frac{\text{var}(L)}{(R-B)^2} + \frac{(L-B)^2.\text{var}(R)}{(R-B)^4} + \frac{(R-L)^2.\text{var}(B)}{(R-B)^4} \qquad (36)$$

and the standard error (S.E.) is the square root of the variance.

3.5 Calculation of isotopic fractionation

Calculation of an isotopic fractionation factor, ε, during the conversion of a substrate(s) to a product (p) depends on the system being measured. In a closed system where the substrate is being depleted, $\varepsilon_{p/s}$ is calculated by the following expression (Mariotti et al. 1981):

$$\varepsilon_{p/s} = (\delta^{15}N_{s,o} - \delta^{15}N_p).\frac{(1-f)}{f.\ln(f)} \qquad (37)$$

where $\delta^{15}N_{s,o}$ and $\delta^{15}N_p$ are the ^{15}N concentrations of the substrate at time 0 and the accumulated product respectively, and f is the unreacted fraction of substrate. This expression applies for a unidirectional reaction, s→p, and it is assumed that $\varepsilon_{p/s}$ is constant with the extent of the reaction. However, where the substrate can be considered as an infinite reservoir with respect to the quantity of

the product formed, f approaches 1 and the expression (1-f)/f.ln f approaches -1, and expression (37) simplifies to:

$$\varepsilon_{p/s} \cong \delta^{15}N_p - \delta^{15}N_s. \qquad (38)$$

More strictly,

$$\varepsilon_{p/s} = \frac{\delta^{15}N_p - \delta^{15}N_s}{\delta^{15}N_s + 1000} \cdot 1000 \qquad (39)$$

although equation (38) is sufficiently accurate if $\delta^{15}N_s$ is small relative to 1000.

In the study on isotopic fraction during N_2 fixation (Chapter 5), the substrate (atmospheric N_2) was infinite, $\delta^{15}N_s = 0$ (if values are given with respect to atmospheric N_2) and equation (38) was used to estimate the isotopic fractionation factor. Data are presented as the $\delta^{15}N$ (with respect to atmospheric N_2) of the legume N grown with atmospheric N_2 as the sole source of N and are designated B (see section 3.4).

In the study on isotopic fractionation during assimilation of nitrate or nitrite by nitrate reductase or nitrite reductase respectively (Chapter 6), high substrate concentrations were generally used and therefore equation (38) was also used in calculating an isotopic fractionation factor, unless otherwise stated.

CHAPTER 4

VARIATIONS IN THE NATURAL ENRICHMENT OF ^{15}N IN THE PROFILES OF PASTURE SOILS

4.1 Introduction

Total soil nitrogen is generally enriched in the stable isotope of nitrogen, ^{15}N, relative to atmospheric N_2 (Delwiche and Steyn 1970; Karamonos et al. 1981) but for unknown reasons, the enrichment may vary within individual soil profiles (Delwiche and Steyn 1970; Virginia 1980; Steele et al. 1981) and over large areas of virgin and cultivated soils (Rennie et al. 1976; Broadbent et al. 1980).

It has been suggested that differences in the natural abundance of ^{15}N between different soils may reflect differences in N cycling processes (Rennie et al. 1976; Mariotti 1982). Thus, Virginia (1980) found that the ^{15}N concentration of total soil N increased with plant succession, being lowest in young sandy soils. If relationships such as this could be established for soils on which crop and pasture legumes are grown, it might be possible to recognise those soils with a high natural abundance of ^{15}N where accurate estimates of N_2 fixation by legumes could be obtained.

In using the natural abundance of ^{15}N for estimating N_2 fixation by legumes it is the ^{15}N concentration of the plant-available fraction of the soil N which is important, and this concentration is frequently different from that in the total soil N (Bremner and Tabatabai 1973; Feigin et al. 1974a; Black and Waring 1977). The natural abundance of ^{15}N in the plant-available fraction of the soil N is most easily measured by the ^{15}N abundance in the total N of a reference plant which obtains all of its N from the soil. However, it is important

that this reference plant absorbs N which accurately represents the isotopic composition of the soil N absorbed by the legume. This might not be true when there is an appreciable variation in the isotopic composition of soil N laterally across the soil surface, or vertically with depth in the soil profile, unless the root geometry and N uptake patterns of the two plants are comparable.

The aim of the present study was to examine the variability in the isotopic composition of N in a range of surface soils and soil profiles under native and improved pastures in south-eastern Australia and the possible causes of this variation. The relationship between the ^{15}N concentration of N in several soil fractions, including the plant-available fraction, was also examined.

4.2 Experimental

4.2.1 Lateral variability in $\delta^{15}N$ of surface soils

4.2.1.1 Within a small area

This preliminary study used an apparently uniform site (soil 1, Table 3.1) near Canberra, Australian Capital Territory. The site was chosen from within a pasture of phalaris and subterranean clover which had been lightly grazed by sheep. Soil cores (100 mm diameter, 75 mm depth) were taken at 1 m spacings on a 3 x 4 m grid and analysed for total N and ^{15}N.

4.2.1.2 Within a large catchment

The Pejar Dam catchment (about 400 km^2) near Goulburn in New South Wales was selected to examine variability of total N and $\delta^{15}N$ in the soil, as influenced by parent material and period under subterranean clover pasture. Within soils derived from basaltic,

granitic and sedimentary parent materials (soils 2,3,4, Table 3.1), sites growing both native pastures and pastures improved by applying phosphatic fertilizer and growing subterranean clover for 25 or 55 years were chosen. The surface (0-50 mm) layer of soil was sampled from eighteen of these sites.

4.2.2 Variability in $\delta^{15}N$ with soil depth

4.2.2.1 Total soil N

Soils from nine sites within the Pejar Dam catchment were further examined for changes in total N and $\delta^{15}N$ with depth. The 0-50, 50-100, 100-200, 200-300, 300-400, 400-500 and 500-600 mm layers of soil were sampled. In addition, samples of the parent rock, from which each of the soils were derived, were collected, crushed, finely ground and analysed for total N and ^{15}N.

4.2.2.2 Soil fractions

The forms and distribution of organic N within soil profiles were examined by chemical, physical and biological techniques.

4.2.2.2.1 Chemical and physical fractionation

The soils chosen for this study were derived from granite or basalt (soils 2 and 4, Table 3.1) and were taken from beneath native pasture or 55 year old subterranean clover pasture. Only the 0-50, 100-200 and 500-600 mm layers were used.

Soil organic matter was chemically fractionated into fulvic acid, humic acid and humin as described in section 3.2.2.5. Soils were also physically fractionated into three particle size fractions (< 2, 2-53, > 53 μm) as described in section 3.2.2.6.

4.2.2.2.2 Fractionation of biologically significant nitrogen

A yellow podzolic soil (soil 5, Table 3.1) was separated into 0-100, 100-200, 200-300 and 300-600 mm layers, and soil from each layer was passed through a 1 mm sieve and mixed. The initial inorganic N, mineralizable N and plant-extractable N were determined for each soil layer and the isotopic compositions compared with that of total N.

Initial inorganic N: About 24 hours after collection of soil from the field, samples from each layer were analysed for inorganic N.

Mineralizable N: Soil samples (300 g) from each of the four depths were placed in 2 l jars and the moisture content adjusted to 60% of field capacity with a fine mist of water. Jars were covered to reduce water loss and placed in an incubator at 25°C. Aerobic conditions were maintained by regular opening of the jars and the soil moisture was maintained at about 60% of field capacity by regular watering. After 55 days incubation, mineralized N was extracted and analysed for inorganic N and ^{15}N.

Plant-extractable N: This was measured in pot experiment 1 using five replicate samples of soil from each of the four layers. The soil (2 kg) was mixed with 1 g P as superphosphate and filled into a 150 mm diameter pot fitted with a polyethylene liner. The moisture content of the soil was adjusted to 75% of field capacity and maintained near this level by daily watering. Seeds of annual ryegrass were sown into each pot and thinned to seven plants per pot after emergence. In addition, phalaris seeds were sown into pots containing the 0-100 mm layer of soil. Fifty-five days after sowing, plant shoots were harvested by cutting at the soil surface and roots were recovered from the soil by sieving and washing. Plant material was analysed for total N and ^{15}N.

4.2.3 Effect of $\delta^{15}N$ of soil nitrogen on the error in estimating P

The effect of the value for the $\delta^{15}N$ of plant-available soil N on the error in estimating P using the natural ^{15}N abundance method was examined using equation 36 (Chapter 3) for the S.E. of P. A range of P values was used to examine the effect of P on the relationship between the S.E. of P and the $\delta^{15}N$ of plant-available soil N. For a fixed P value, the $\delta^{15}N$ of the legume was examined by rearranging equation 31 (Chapter 3) to give:

$$\delta^{15}N_{legume} = \delta^{15}N_{SN} - [P \times (\delta^{15}N_{SN} - B)] \qquad (40)$$

where SN is the plant-available soil N and B is the $\delta^{15}N$ of legume N derived entirely from atmospheric N_2. In these calculations, B was taken as +0.59 (±0.2)‰ (see Table 5.1 for subterranean clover) and a value of 0.2‰ was used for the S.E. of the $\delta^{15}N$ of plant-available soil N and of the $\delta^{15}N$ of legume N. This value is typical of that found in the field experiment (see Table 9.3).

4.3 Results

4.3.1 Lateral variability in $\delta^{15}N$ of surface soils

4.3.1.1 Within a small area

The concentration of total N in the surface soil from a 12 m^2 grid ranged from 0.218 to 0.285% and averaged 0.245% (S.E. = 0.004) and the $\delta^{15}N$ of that N ranged from 5.01 to 7.95‰ and averaged 6.31‰ (S.E. = 0.23) . There was no directional trend in either concentration or $\delta^{15}N$ of total N within the grid.

4.3.1.2 Within a large catchment

The concentration of total N in pasture soils (0-50 mm) from the Pejar Dam catchment ranged from 0.22 to 0.42% N and averaged 0.285% (S.E. = 0.016), and the $\delta^{15}N$ ranged from 2.55 to 6.79‰, with a mean of 5.16‰ (S.E. = 0.27).

There was no significant relationship between $\delta^{15}N$ and soil parent material. The concentration of total N generally increased with greater periods of pasture improvement but there was no corresponding effect on $\delta^{15}N$ (Fig. 4.1).

4.3.2 Variability in $\delta^{15}N$ with soil depth

4.3.2.1 Total N of soils and parent rocks

Figure 4.2 shows the changes in concentration and $\delta^{15}N$ of the total N in two granitic soil profiles from native and 55 year old subterranean pastures (soil 2, Table 3.1). The results are typical of all the soil profiles examined. A decrease in total N concentration and an increase in $\delta^{15}N$ with depth occurred in both virgin and improved soils. Within all profiles the increase in $\delta^{15}N$ was most marked in the 0-300 mm layer and little change occurred in the 300-600 mm layer. The $\delta^{15}N$ values for the 0-50 mm layer of the nine soils ranged from 2.77 to 6.12‰ while those from the 500-600 mm layer ranged from 7.16 to 10.06‰.

The concentration of N in the basalt and granite parent rocks was very low relative to that in the sedimentary rock and showed no relationship with the $\delta^{15}N$ (Table 4.1). The $\delta^{15}N$ of the total N in the parent rocks was substantially lower than the values observed for the 500-600 mm soil layers for all soil examined from the catchment.

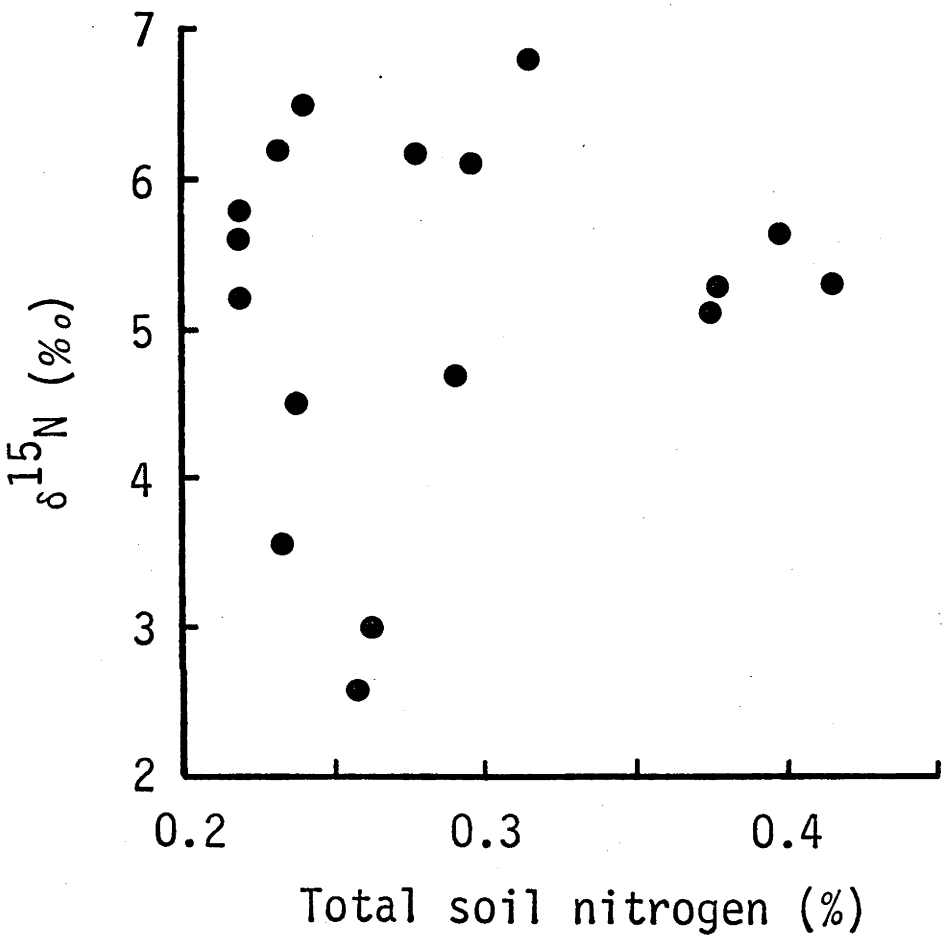


Figure 4.1. Variation in concentration and $\delta^{15}N$ of total nitrogen in soils (0-50 mm depth) sampled from the Pejar Dam catchment, New South Wales.

Figure 4.2. Variation in concentration (△ — —△) and $\delta^{15}N$ (●——●) of total nitrogen with depth in soils derived from granite under (a) native pasture and (b) 55-year-old subterranean clover pasture. Each value is the mean of three replicates and bars represent averages for ± 1 S.E.

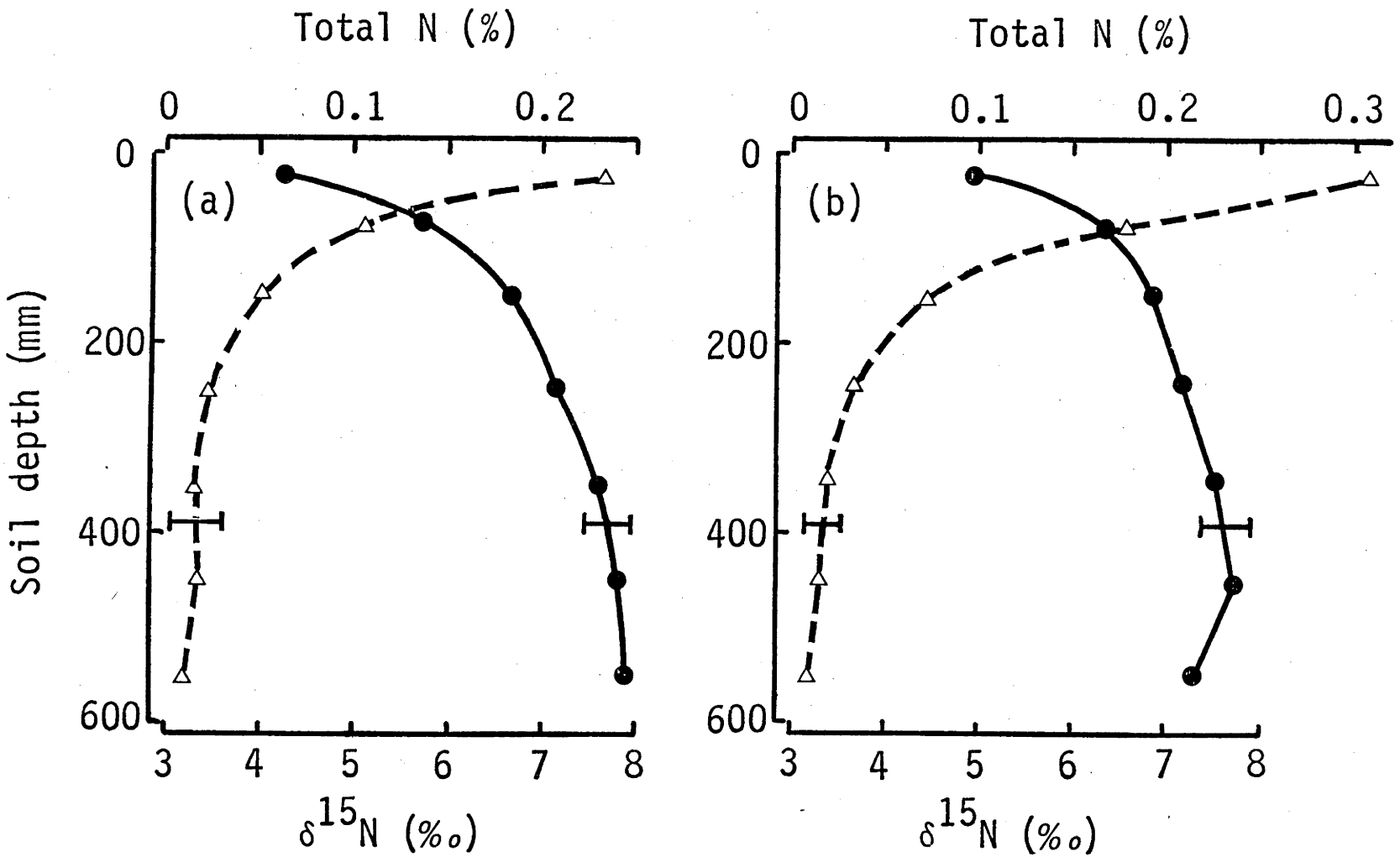

Total N (%)
0
0.1
0.2
0.3
(a)
(b)
Soil depth (mm)
0
200
400
600
3
4
5
6
7
8
δ15N (‰)

Table 4.1. Concentration and $\delta^{15}N$ of total N in basalt, granite and sedimentary rocks. Each value is the mean of three replicates and values in brackets are standard errors.

Parent rock	Total N $\mu g\ Ng^{-1}$	$\delta^{15}N$ ‰
Basalt	27.3 (10.8)	1.81 (0.14)
Granite	41.7 (15.0)	5.30 (0.92)
Sedimentary	261 (106)	3.90 (1.34)

4.3.2.2 Chemical and physical fractions of soils

Chemical separation of organic N revealed that most of the N occurred in the humin fraction, and that pasture improvement generally increased the N concentration in all fractions in all soil layers except the 500-600 mm layer (Table 4.2). There was a greater proportional increase in humic acid with pasture improvement than in the other two fractions in the granitic soils, but this did not occur with the basaltic soils (Table 4.2).

There were no consistent differences in $\delta^{15}N$ between the three fractions in the different layers of both soils with varying time under subterranean clover (Table 4.2). The $\delta^{15}N$ of each of the chemical fractions increased down the profile in the same way as total soil N (compare Table 4.2 and Fig. 4.2).

Separation of the soil particles revealed that most of the N occurred in the clay-sized fraction (<2 μm). In addition, the proportion of N in the clay-sized fraction in the 500-600 mm layer of both soils was greater than in the corresponding fraction of the 0-50 and 100-200 mm layers (Table 4.3). The concentration of N in each of the particle size fractions increased with greater periods of pasture improvement in the 0-50 and 100-200 mm layers of both soils, with the largest proportional increase occurring in the sand-sized fraction (Tables 4.3 and 4.4). In all soils, the total N was fairly evenly distributed among the three fractions in the 0-50 mm layer, whereas at the 500-600 mm depth the N was predominantly in the clay-sized fraction (Tables 4.3 and 4.4).

In both soils there was an increase in $\delta^{15}N$ with decreasing particle size for each soil layer, (the value for the clay-sized fraction being about double that for the sand-sized fractions). In contrast

Table 4.2. Concentration and $\delta^{15}N$ of total N in organic fractions from granitic and basaltic soil profiles supporting native pasture or pastures improved by a period under subterranean clover.

Pasture treatment	Soil depth (mm)	Total nitrogen ($\mu g\ N\ g^{-1}$ oven-dry soil)			$\delta^{15}N$ (‰)			
		Fulvic acid	Humic acid	Humin	Fulvic acid	Humic acid	Humin	S.E.D.[2]
GRANITIC SOIL[1]								
Native	0-50	192	150	1820	5.40	4.68	4.10	
	100-200	68	78	412	5.64	6.14	5.61	0.37
	500-600	35	12	186	6.61	9.11	7.99	
Improved	0-50	252	525	2600	6.37	5.76	5.96	
(55 years)	100-200	107	229	383	6.45	5.98	6.67	0.41
	500-600	26	12	105	7.03	7.94	6.46	
BASALTIC SOIL:								
Improved	0-50	214	364	1538	5.23	5.51	5.74	
(25 years)	100-200	105	218	526	5.85	6.34	6.33	0.32
	500-600	67	76	288	6.55	7.00	6.84	
Improved	0-50	263	459	2098	6.04	5.79	5.92	
(55 years)	100-200	116	251	605	6.48	6.91	7.29	0.43
	500-600	40	24	343	8.93	9.16	8.78	

[1] Terms as defined by Kononova (1966).

[2] Standard error of the difference between any two of the nine means (3 replicates).

Table 4.3. Concentration, distribution and $\delta^{15}N$ of total N in particle size fractions of soil profiles. The soils were derived from granite and supported native pasture or 55 year old subterranean clover pasture.

Parameter	Size fraction	Native pasture 0-50[1]	Native pasture 100-200	Native pasture 500-600	Improved pasture 0-50	Improved pasture 100-200	Improved pasture 500-600
Particle size distribution (% of total)	sand[2]	58	56	45	62	59	55
	silt[3]	36	38	34	32	36	35
	clay[4]	6	6	21	6	5	10
Nitrogen concentration ($\mu g\ N\ g^{-1}$ soil fraction)	sand	769	102	38	1550	176	28
	silt	2240	527	146	3140	756	114
	clay	14140	4350	629	19130	6990	919
Nitrogen distribution (as % of total N in the layer)	sand	21	11	9	31	14	10
	silt	40	39	25	34	38	26
	clay	39	50	66	37	48	64
$\delta^{15}N$ (‰)	sand	3.04	3.46	4.68	4.19	4.57	5.99
	silt	4.50	5.00	6.54	5.06	5.34	6.30
	clay	7.65	8.10	9.01	7.79	7.98	8.04
	S.E.D.[5]		0.42			0.46	

[1] Soil depth in mm.

[2] Sand 2000-50 μm.

[3] Silt 50-2 μm.

[4] Clay <2 μm.

[5] Standard error of the difference between any two of the nine means (3 replicates).

Table 4.4. Concentration, distribution and $\delta^{15}N$ of total N in particle size fractions of soil profiles. The soils were derived from basalt and supported 25 or 55 year old subterranean clover pasture.

Parameter	Size fraction	25 yr old pasture 0-50[1]	100-200	500-600	55 yr old pasture 0-50	100-200	500-600
Particle size distribution (% of total)	sand[2]	16	14	19	23	19	17
	silt[3]	74	72	50	66	68	54
	clay[4]	10	14	31	11	13	29
Nitrogen concentration ($\mu g\ N\ g^{-1}$ soil fraction)	sand	2381	557	218	3497	668	172
	silt	1221	574	270	1059	796	164
	clay	7382	3467	986	8065	3669	821
Nitrogen distribution (as % of total N in the layer)	sand	19	8	9	34	11	10
	silt	45	42	28	29	47	29
	clay	36	50	63	37	42	61
$\delta^{15}N$ (‰)	sand	3.29	3.96	4.99	4.84	5.04	5.64
	silt	5.15	5.66	5.66	5.71	6.96	7.52
	clay	7.29	7.69	8.00	8.80	9.03	9.80
	S.E.D.[5]		0.37			0.49	

[1]Soil depth in mm.

[2]Sand 2000-50 µm.

[3]Silt 50-2 µm.

[4]Clay <2 µm.

[5]Standard error of the difference between any two of the nine means (3 replicates).

there was much less difference in $\delta^{15}N$ between the soil depths within one particle size fraction (a difference of about 30% between the 0-50 and 500-600 mm layers).

4.3.2.3 Biologically-significant nitrogen

When ryegrass was used to extract N from the soil, the amounts obtained decreased with increasing soil depth (Table 4.5). In the 0-100 mm layer of soil, most ryegrass N occurred in the shoots, but as the soil depth increased, the amount of N in the roots relative to the shoots increased. Similarly, in the 0-100 mm layer there was a large difference in the $\delta^{15}N$ of ryegrass N between shoots and roots and this difference decreased with soil depth (Table 4.5). However, in all cases the $\delta^{15}N$ of N in plant shoots was significantly lower than that in the roots. Phalaris roots assimilated less N than ryegrass roots when grown in the 0-100 mm layer and the $\delta^{15}N$ of N in the roots of phalaris was significantly lower than that of ryegrass (Table 4.5). The effect of such differences in ^{15}N concentration between plant parts on the estimate of P will be discussed in detail in Chapter 9.

When total plant (shoots + roots) N was compared with other soil N fractions of biological importance, differences in $\delta^{15}N$ values were obtained (Fig. 4.3). The $\delta^{15}N$ values could be ranked in the following order: initial inorganic N < mineralizable N = plant-extractable N < total soil N, for all soil layers (Fig. 4.3). The $\delta^{15}N$ of the initial inorganic N in all soil layers was close to, but significantly higher than zero, whereas that for the inorganic N mineralized during 55 days incubation was substantially greater than the initial inorganic N.

The $\delta^{15}N$ of total soil N increased with depth, particularly over the 0-200 mm zone, as already noted for the catchment study (Fig.

Table 4.5. Amounts and $\delta^{15}N$ of nitrogen assimilated by ryegrass from different soil layers. Values were compared with those assimilated by phalaris from the 0-100 mm soil layer. Each value is the mean of five replicates (pot experiment 1).

Soil layer (mm)	Grass	Grass N (mg pot^{-1}) shoots	roots	$\delta^{15}N$ (‰)[1] of grass N shoots	roots	S.E.D.
0-100	Ryegrass	96.4	21.8	2.10	4.27	0.17
100-200	Ryegrass	23.8	12.1	3.43	4.99	0.18
200-300	Ryegrass	11.9	6.1	3.02	3.67	0.18
300-600	Ryegrass	7.1	4.5	2.59	3.23	0.15
0-100	Phalaris	94.4	15.5	1.90	3.58	0.13
	S.E.D.	1.5	1.3	0.14	0.17	

[1] With respect to atmosphere N_2.

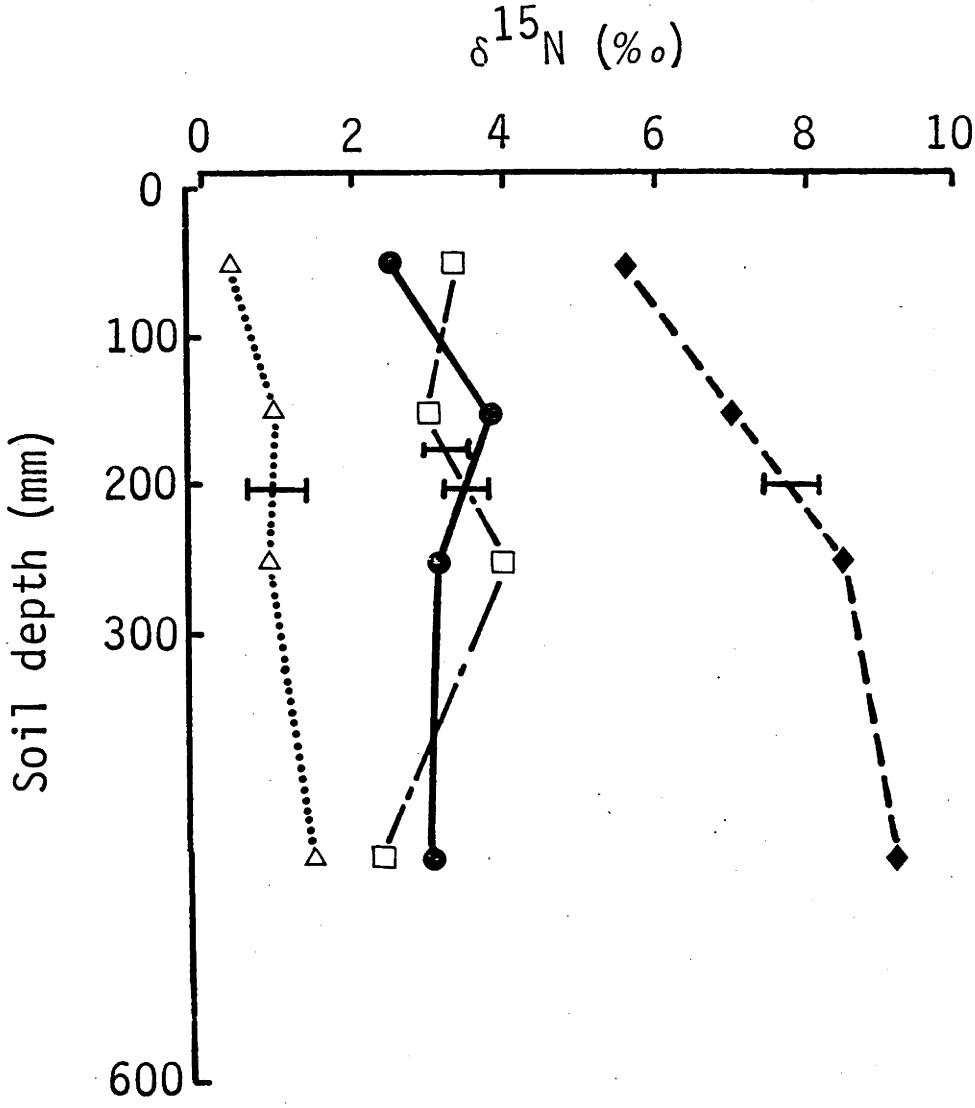


Figure 4.3. Variation in $\delta^{15}N$ of plant and soil fractions; inorganic nitrogen at day 0 (△·····△), inorganic nitrogen mineralized over 55 days (□—-□), plant-extractable nitrogen after 55 days (●——●), and total nitrogen in soil (◆——◆) (pot experiment 1). Each value is the mean of five replicates and bars represent averages for ± 1 S.E.

4.2). However, the $\delta^{15}N$ of the mineralized N or plant N did not show any obvious trends with soil depth.

4.3.3 Effect of $\delta^{15}N$ of soil nitrogen on the error in estimating P

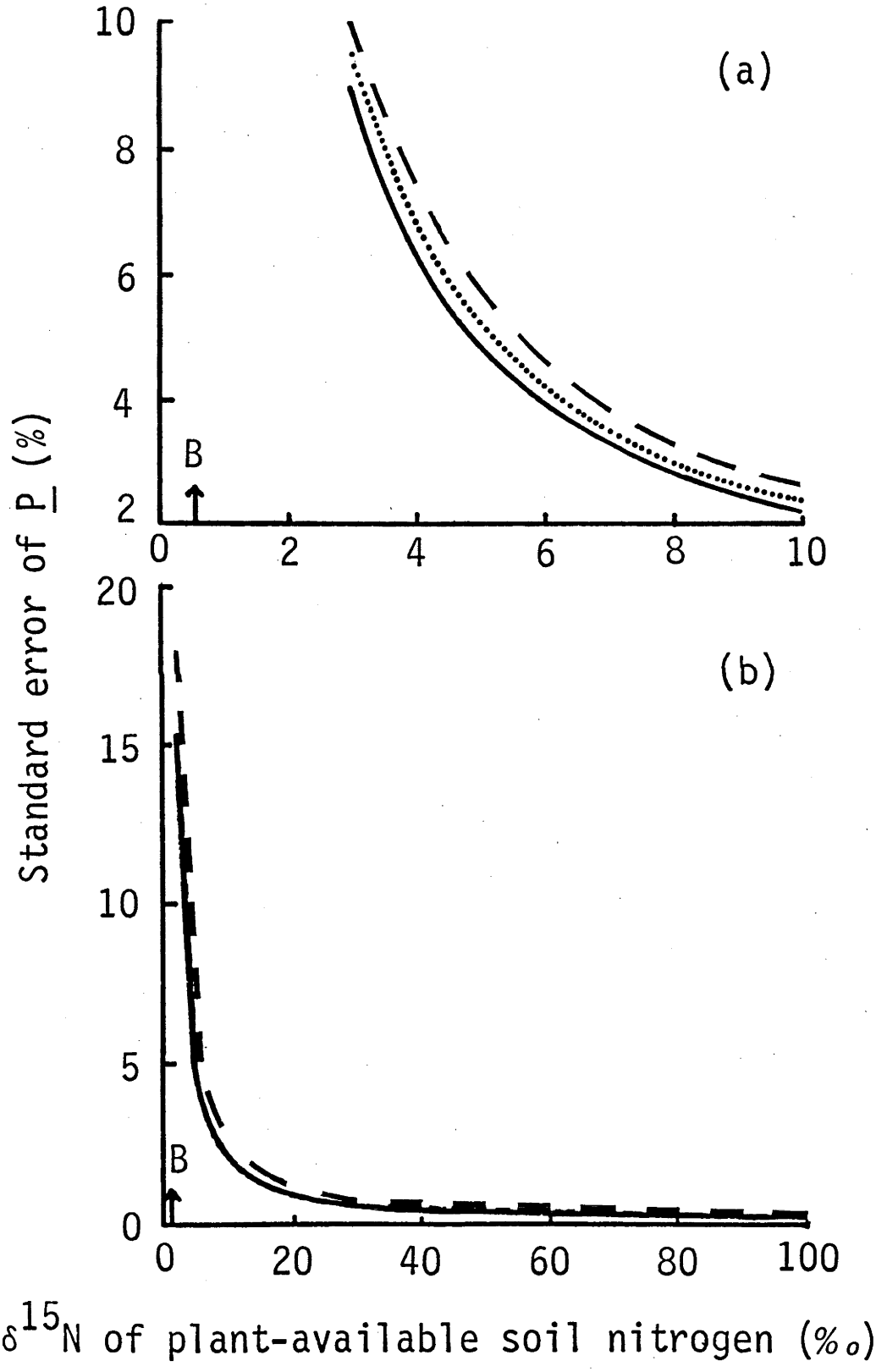
These calculations revealed that the value for the $\delta^{15}N$ of plant-available soil N has a large effect on the error obtained in estimating P by the natural ^{15}N abundance method. As the $\delta^{15}N$ of plant-available soil N approached that of the legume N derived from atmospheric N_2, there was an exponential increase in the S.E. of P (Fig. 4.4). Thus, to obtain a value for the S.E. of P of < 5% it is necessary to have a $\delta^{15}N$ value for plant-available soil N of > 4.5‰. There was little effect of the P value on the relationship between the $\delta^{15}N$ of plant-available soil N and the S.E. of P (Fig. 4.4).

4.4 Discussion

4.4.1 Variations in $\delta^{15}N$ of surface soils

The range in $\delta^{15}N$ for total N in pasture soil (0-50 mm) taken from an apparently 'uniform' area of 12 m^2 was 69% of that from a 400 km^2 catchment which covered large differences in soil and pasture types. Karamanos et al. (1981) reported a similar range in the spatial variability of $\delta^{15}N$ in total N from native pasture soils at three locations (each comprising 60 ha) in Canada. In a review on the spatial variability of total N in soils, Biggar (1978) concluded that up to one half of the variability in a field can occur within any one m^2 of the field. It would appear that this also applies for the natural abundance of ^{15}N. However, this random variation does not negate the value of natural ^{15}N abundance in N_2 fixation studies with small plots because all $\delta^{15}N$ values were significantly ($P<0.01$) higher than that of atmospheric N_2.

Figure 4.4. Effect of $\delta^{15}N$ of plant-available soil nitrogen on the error in estimating the proportion ($\underline{P}$) of legume nitrogen fixed. Calculations were based on data for subterranean clover. Data are for $\underline{P}$ values of 90 (— —), 75 (·····) or 50 (——) % and a) is an enlargement of that section of b) in which the $\delta^{15}N$ of plant-available soil nitrogen usually occurs.


(a)
(b)
B
B
Standard error of P (%)
δ15N of plant-available soil nitrogen (‰)
0
2
4
6
8
10
0
5
10
15
20
0
20
40
60
80
100

Within the large catchment the range in $\delta^{15}N$ values is close to that reported by Black and Waring (1977) for three Queensland soils (2.9 to 6.7‰) of a different soil group and is similar to that obtained for soils in other countries (see Table 2.4). The reasons for variations in the $\delta^{15}N$ of total N in surface soils within the large catchment were not obvious. The $\delta^{15}N$ showed no relationship with soil parent material, despite some differences in $\delta^{15}N$ of total N in the parent rock (Table 4.1). This suggests that accumulation and transformation of organic N may be the main processes responsible for the variations in $\delta^{15}N$ of total N in surface soils. There was an accumulation of organic N in the surface soil with increasing time under subterranean clover pasture, presumably due to fixation of atmospheric N_2 and subsequent incorporation into the soil organic matter (Donald and Williams 1954). However, the $\delta^{15}N$ of surface soil N did not decrease with time under subterranean clover as might be expected from large additions of atmospherically-fixed N. With an average rate of N_2 fixation of 100 kg N ha^{-1} $year^{-1}$ which is a feasible estimate (J.R. Simpson, unpublished), the $\delta^{15}N$ of total N in the 0-200 mm layer of the virgin soil (Fig. 4.2; mean value for the top three soil depths weighted for total soil N) should have decreased over 55 years of pasture improvement from 5.21 to 1.91‰ if all of the fixed N (with a $\delta^{15}N$ of 0.59‰, see Table 5.1) had been incorporated into the soil organic matter. In fact, in this soil the $\delta^{15}N$ value had increased to 5.76‰. During the 55 years of pasture improvement 911 kg N ha^{-1} had accumulated in the 0-200 mm layer and even if this was the total amount fixed then a $\delta^{15}N$ value of 3.84‰ would have been expected. It would appear from these observations that large losses of fixed N have occurred and that these losses have resulted

in considerable isotopic fractionation. Denmead et al. (1974) have shown that large losses of N from a grazed pasture can occur by ammonia volatilization and it has been shown that fractionation of isotopic N occurs during volatilization (Haurat et al. 1981; Turner et al. 1983) resulting in the residual N being enriched in ^{15}N relative to the original substrate (Farquhar et al. 1983). Other transformations and transfers such as mineralization, denitrification and leaching can also cause ^{15}N enrichment of the residual organic N (Delwiche and Steyn 1970; Blackmer and Bremner 1977; Mariotti 1982). Thus it would appear that the net effect of these transformations of fixed N in the soil is to maintain a relatively constant value for $\delta^{15}N$ of total N in the surface soil.

4.4.2 Variability in $\delta^{15}N$ with soil depth

All soil profiles showed an increase in $\delta^{15}N$ with depth. The $\delta^{15}N$ within a soil profile may be influenced by the $\delta^{15}N$ of the parent rock, particularly deep in the profile where added organic N is small. However, values for $\delta^{15}N$ of total N in parent rock from the Pejar Dam catchment were substantially lower than the values for the 500-600 mm depth of all soils from this area. This indicates that soil forming and other processes have substantially enriched the soil profile in ^{15}N compared with the parent rock.

Analysis based on particle size within soil profiles gave some indication of the processes which may be responsible for the change in $\delta^{15}N$ with depth. It is believed that the sand-sized fraction contains vegetative debris (e.g. litter and roots), the clay-sized fraction contains humic substances largely associated with the clay minerals and the silt-sized fraction, being intermediate, contains complexes of debris and humus (see for example Mariotti 1982).

Fractionation of isotopic N during mineralization (Delwiche and Steyn 1970) of recently added organic N leads to an increase in the $\delta^{15}N$ of N in the residual humus. Hence the more humified clay-sized fraction has a higher $\delta^{15}N$ than the sand-sized fraction as also found by Mariotti (1982). The proportion of total N in this clay-sized fraction increased with soil depth, resulting in an increase in the $\delta^{15}N$ of the total organic N with depth (Fig. 4.3). This increase in $\delta^{15}N$ of total N with depth will be accentuated by the movement of organic N down the profile with time because this movement is likely to be greatest with the smaller, clay-sized organic fraction (as shown in Tables 4.3 and 4.4), either moving separately as chelates or in combination with clay mineral particles (e.g. by illuviation).

4.4.3 $\delta^{15}N$ of mineralizable and plant-extractable N

Most agronomic studies are concerned with plant-extractable N and therefore the relationship between this and other soil N fractions is important. In the present study, the $\delta^{15}N$ of inorganic N and plant N was less than that of total N. The $\delta^{15}N$ of the inorganic N initially present in the four soil layers was close to zero but the $\delta^{15}N$ of this fraction increased during incubation. Feigin *et al.* (1974a) found that the $\delta^{15}N$ of mineralized N increased with time and became constant after about five weeks incubation. The low initial values for $\delta^{15}N$ of inorganic N may be an artifact due to the disturbance of soil during collection and comminution. Whatever the reason, the amount of inorganic N present initially was small relative to that present after 55 days incubation and therefore did not affect the $\delta^{15}N$ value of the mineralized N. There was no difference between the $\delta^{15}N$ of plant N and inorganic N released during incubation, indicating that the latter may serve as an index of the $\delta^{15}N$ in plant-available N.

Although the $\delta^{15}N$ of total soil N increased with depth there was no change in the $\delta^{15}N$ of plant-extractable N with depth. The $\delta^{15}N$ of plant-extractable N was also consistently lower than that for the total soil N (Fig. 4.3). These findings support the suggestion made above that the high subsoil values for $\delta^{15}N$ in the total soil N are due to an increase in the proportion of stable humic substances which are high in $\delta^{15}N$, since it has been shown (Ford and Greenland 1968) that the mineralizable fraction of soil organic N is mainly associated with the coarser-particles, not the clay-sized fraction.

The use of the natural ^{15}N abundance method for quantifying N_2 fixation by legumes in the field depends on there being a difference in the $\delta^{15}N$ between soil and atmospheric N (Bardin et al. 1977), and the extent to which they differ can have a marked effect on the precision in estimating P (Fig. 4.4). With this technique, an incorrect estimate of P may be obtained if there is a change in the $\delta^{15}N$ of the plant-extractable soil N within the soil profile, or at least within the zone of root penetration. Thus, Steele et al. (1981) claimed that with the natural ^{15}N abundance method it is important to match the legume and non-N_2-fixing reference plant in their depth of root penetration (or zone of N uptake). However, they based their comments entirely on observations of changes in the $\delta^{15}N$ in the total soil N with depth. The present study shows that the $\delta^{15}N$ of the plant-extractable fraction may vary little with depth. This enhances the feasibility of using natural ^{15}N abundance in field studies on N_2 fixation.

CHAPTER 5

ISOTOPIC FRACTIONATION DURING N_2 FIXATION BY THREE PASTURE LEGUMES

5.1 Introduction

The equations for estimating P by the natural ^{15}N abundance (equation 31, Chapter 3) and ^{15}N isotope dilution (equation 32, Chapter 3) methods contain a value for the ^{15}N concentration of legume N derived from atmospheric N_2. This value is commonly assumed to be the same as that for atmospheric N_2 i.e. 0.3663 ± 0.0004 atoms % ^{15}N (Junk and Svec 1958). Mariotti (1983) found that the isotopic composition of atmospheric N_2 was remarkably constant (standard deviation of ± 9 x 10^{-6} atoms % ^{15}N or $\delta^{15}N$ of ± 0.026 ‰) throughout the world. However, the isotopic composition of N_2-fixing bacteria and crop and pasture legumes grown entirely on atmospheric N_2 can differ significantly from that of atmospheric N_2 (see Table 2.6) due to isotopic fractionation during N_2 fixation. Thus, it is important to establish the level of isotopic fractionation during N_2 fixation by legumes if an accurate estimate of P is to be obtained.

The aim of this study was to obtain values for isotopic fractionation during N_2 fixation by the three major pasture legumes, subterranean clover, white clover and lucerne for use in subsequent studies.

5.2 Experimental

Seeds of subterranean clover (cv. Mount Barker), white clover (cv. N.Z. Grasslands Huia) and lucerne (cv. Hunter River) were surface-sterilized and scarified with concentrated H_2SO_4, and pregerminated in sterile agar at 25°C for one day. About 20 seedlings of each species were planted into free-draining pots (250 mm diameter) containing a pre-washed 1:1 mixture of vermiculite and quartz sand. Eighteen replicates of each of the three species were used.

At planting, an inoculum containing a suspension of Rhizobium trifolii strain TA1 for subterranean clover and white clover, and Rhizobium meliloti strain CC169 for lucerne, was sprayed around each seedling. The plants received regular applications of distilled water and a N-free nutrient solution, containing (mg l^{-1}) KH_2PO_4 (110), KC1 (166), $CaCl_2$ (5.6), $MgSO_4.7H_20$ (111), $FeCl_3$ (14), H_3BO_3 (2.8), $MnSO_4.4H_2O$ (2.1), $ZnSO_4.7H_2O$ (0.2), $CuSO_4.5H_2O$ (0.08), and Na_2MoO_4 (0.11), adjusted to pH 6.8 with NaOH. The plants were grown in a naturally lit glasshouse with temperatures ranging from 18 to 28°C.

Seedlings were planted on 27 September 1981 and harvests were made at 9, 14, 20, 30, 40 and 60 days after planting. Harvests involved removal of adhering growth medium by thorough washing and separation into shoots, roots and nodules (where present). Plant samples were analysed for total N and ^{15}N.

5.3 Results

Despite the marked differences in the N content of the legume seeds there was little difference between species in the plant N at the

final harvest (Fig. 5.1). In all species, the $\delta^{15}N$ of plant N at day 9 was greater than that in the seed (Fig. 5.2). This was most evident with lucerne (Fig. 5.2c). After day 9, the $\delta^{15}N$ decreased with time, apparently reaching an equilibrium after day 30. To overcome the effect of this initial increase in $\delta^{15}N$, the estimates of the isotopic fractionation factor, B, were obtained from the intercepts of the regression lines between $1/\delta^{15}N_{legume}$ and 1/time from day 9 onwards (Bergersen and Turner 1983). Thus, B values for time infinity were obtained and any initial changes in $\delta^{15}N$ became insignificant. Values of B obtained with this method tended to be lower than values for the $\delta^{15}N$ of plant N obtained at days 40 and 60. The latter values (± S.E.) for whole plant data were 0.606 (± 0.081), 0.770 (± 0.095) and 1.228 (±0.120) for subterranean clover, white clover and lucerne respectively. These values, and those in Table 5.1, show that isotopic fractionation during N_2 fixation by lucerne was significantly higher than by subterranean clover or white clover.

There was no significant difference in the $\delta^{15}N$ of N between plant parts for all legumes at all harvests (Fig. 5.2). Thus, there was no significant difference in the B values estimated from data for whole plants or shoots-alone (Table 5.1).

5.4 Discussion

The $\delta^{15}N$ of N in the legumes was found to increase up to day 9 after sowing and then decrease to a relatively constant level. The initial increase was not due to N_2 fixation because nodules had not been formed at that stage of growth. The increase may have been caused by fractionation during remobilization of seed N (in

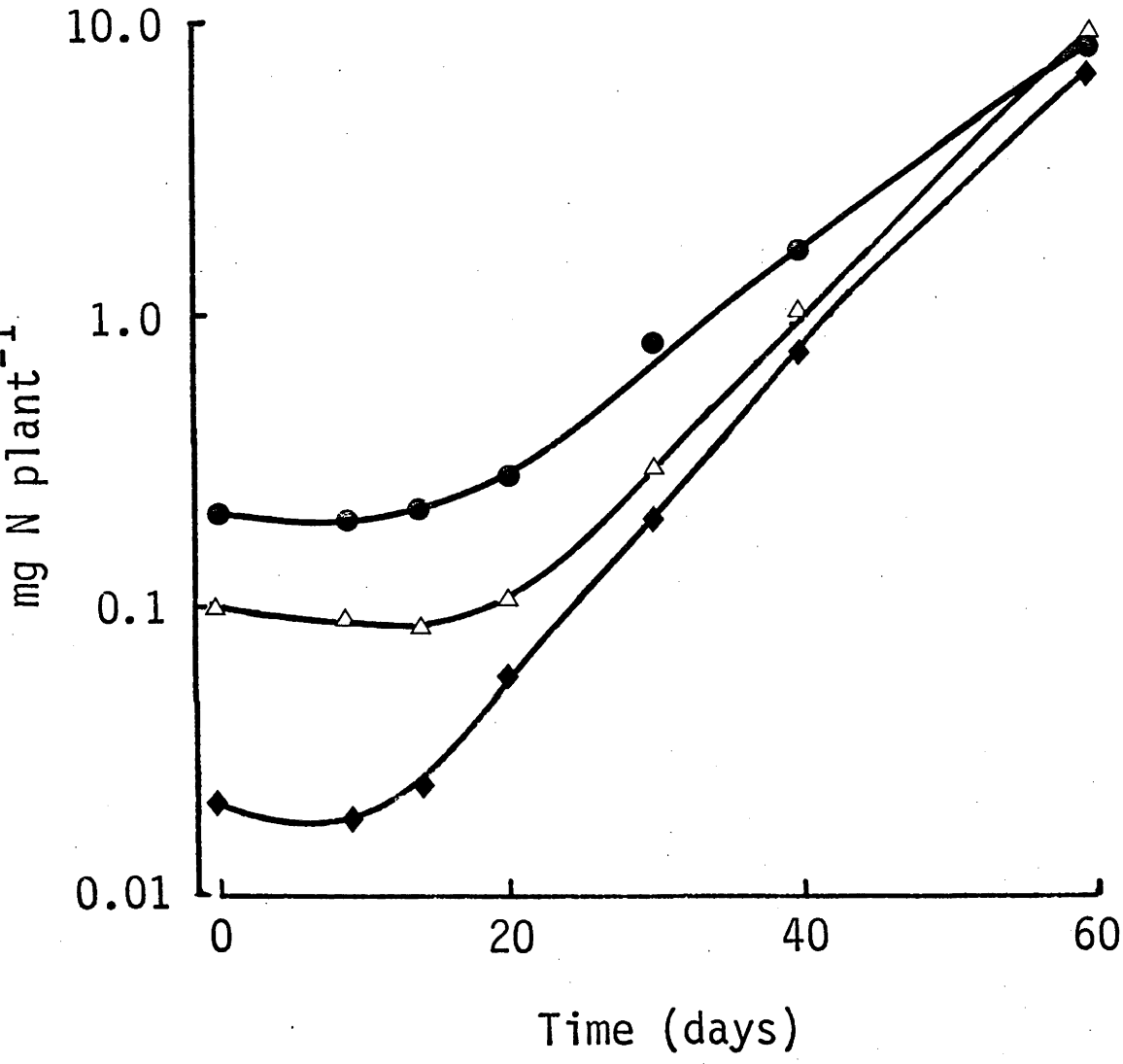


Figure 5.1. The time course of N_2 fixation by subterranean clover (●), white clover (◆) and lucerne (△). Each value is the mean of three replicates and values at zero time represent the nitrogen content of the seed (pot experiment 2).

Figure 5.2. Changes with time in the isotopic composition of nitrogen in (a) subterranean clover, (b) white clover and (c) lucerne grown with atmospheric N_2 as their sole source of nitrogen (pot experiment 2). Values are for shoots (●), roots (○) and nodules (◆). Each value is the mean of three replicates and bars represent S.E.D.'s.

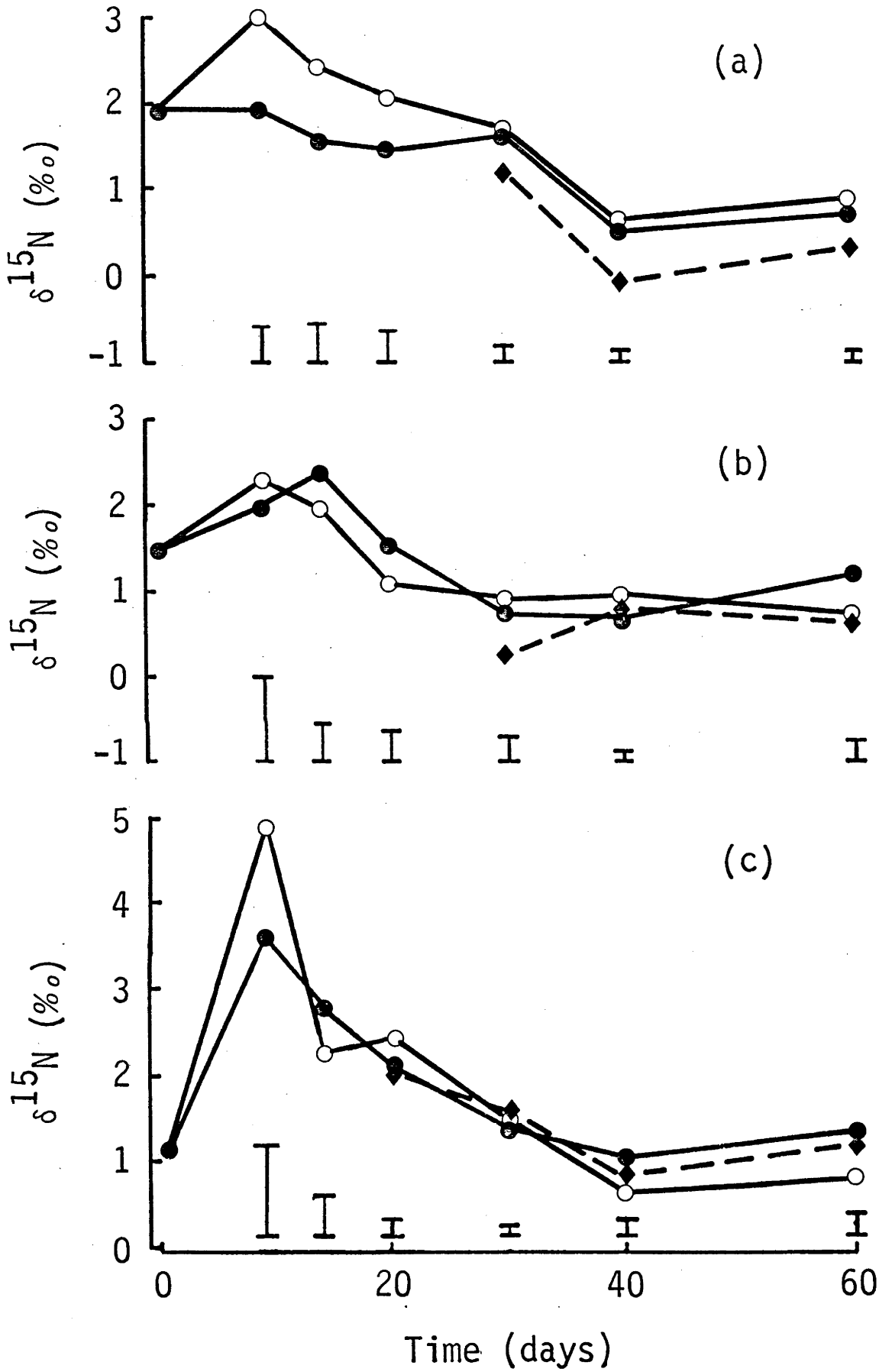

(a)
(b)
(c)
$\delta^{15}N$ (‰)
3
2
1
0
-1
5
4
0
20
40
60
Time (days)

Table 5.1. Effect of legume species on the estimate of the isotopic fractionation factor (B)[1] associated with symbiotic N_2 fixation by subterranean clover, white clover and lucerne (pot experiment 2). Values in brackets are standard errors.

Legume	Shoots only		Whole plants	
	Atoms % ^{15}N	$\delta^{15}N$[2]	Atoms % ^{15}N	$\delta^{15}N$
Subterranean clover	0.36652(8.4×10^{-5})	0.621(0.235)	0.36652(3.9×10^{-5})	0.594(0.111)
White clover	0.36653(8.1×10^{-5})	0.648(0.227)	0.36651(4.1×10^{-5})	0.580(0.116)
Lucerne	0.36670(11.3×10^{-5})	1.090(0.314)	0.36665(4.5×10^{-5})	0.974(0.132)

[1] Estimated as the intercept between $1/\delta^{15}N$ and 1/time, using 18 observations (i.e. three replicates from each of six time periods).

[2] In ‰ with respect to atmospheric N_2.

conjunction with some loss of N), or by the uptake of traces of inorganic N which had not been removed from the growth medium by the purification process used (see Bergersen and Turner 1983).

Legumes were found to differ in their level of isotopic fractionation during N_2 fixation, as was found by other workers (e.g. Kohl and Shearer 1980; Mariotti et al. 1980). These differences may be a feature of the host plant or they may be due to the different Rhizobium used. Rhizobium trifolii strain TA1 was used for both white and subterranean clovers and their B values were the same. Conversely, Steele et al. (1983) measured relatively large differences in isotopic fractionation during N_2 fixation by white clover using different strains of R. trifolii.

The B value obtained for subterranean clover was lower than that obtained by Bergersen and Turner (1983) for the same species. This may also be due to differences in the strain of R. trifolii used (TA1 vs. WU95) or to differences in growth conditions. Different estimates of B for the same legume species have also been obtained by other workers (see Table 2.6); negative B values were obtained in studies in a French laboratory (Amarger et al. 1977; Amarger et al. 1979; Mariotti et al. 1980) and positive values were found in studies in a U.S.A. laboratory (Kohl and Shearer 1980). The fact that these differences between laboratories may be due to differences in isotopic fractionation during sample preparation cannot be overlooked. However, if such fractionation had occurred, it may not affect the estimation of P when equation 31 is used if it was consistent throughout all analyses, because this equation is based entirely on differences between measured values of $\delta^{15}N$.

The value of B can affect the accuracy of estimation of P by the natural ^{15}N abundance method. As the value of B increases, the difference between B and the $\delta^{15}N$ of plant-available soil N decreases and the error in estimating P by the simplified equation (21, Chapter 2) is increased. This may be likened to a shift to the left of the curves in Fig. 4.4. Also, for a fixed B value, the error in estimation of P increases as the $\delta^{15}N$ of the plant-available soil N (shown as $\delta^{15}N_R$ in Fig. 5.3) decreases, and as the value of P increases (Fig. 5.3). Thus, B has a negligible effect on the estimation of P where the P value is low and/or where the difference between B and the $\delta^{15}N$ of soil-derived N exceeds 10 $\delta^{15}N$ units. With the ^{15}N isotope dilution method this difference is artificially enlarged and therefore the estimation of P by equations 31 and 32 (Chapter 3), which include B values, will not be significantly different from that by the more simplified equations (15 and 21, Chapter 2) where it is assumed that there is no isotopic fractionation during N_2 fixation. Nevertheless, the B values obtained from the intercepts of the reciprocals of $\delta^{15}N$ and time (Table 5.1) were used in all subsequent estimates of P by natural ^{15}N abundance and ^{15}N isotope dilution methods.

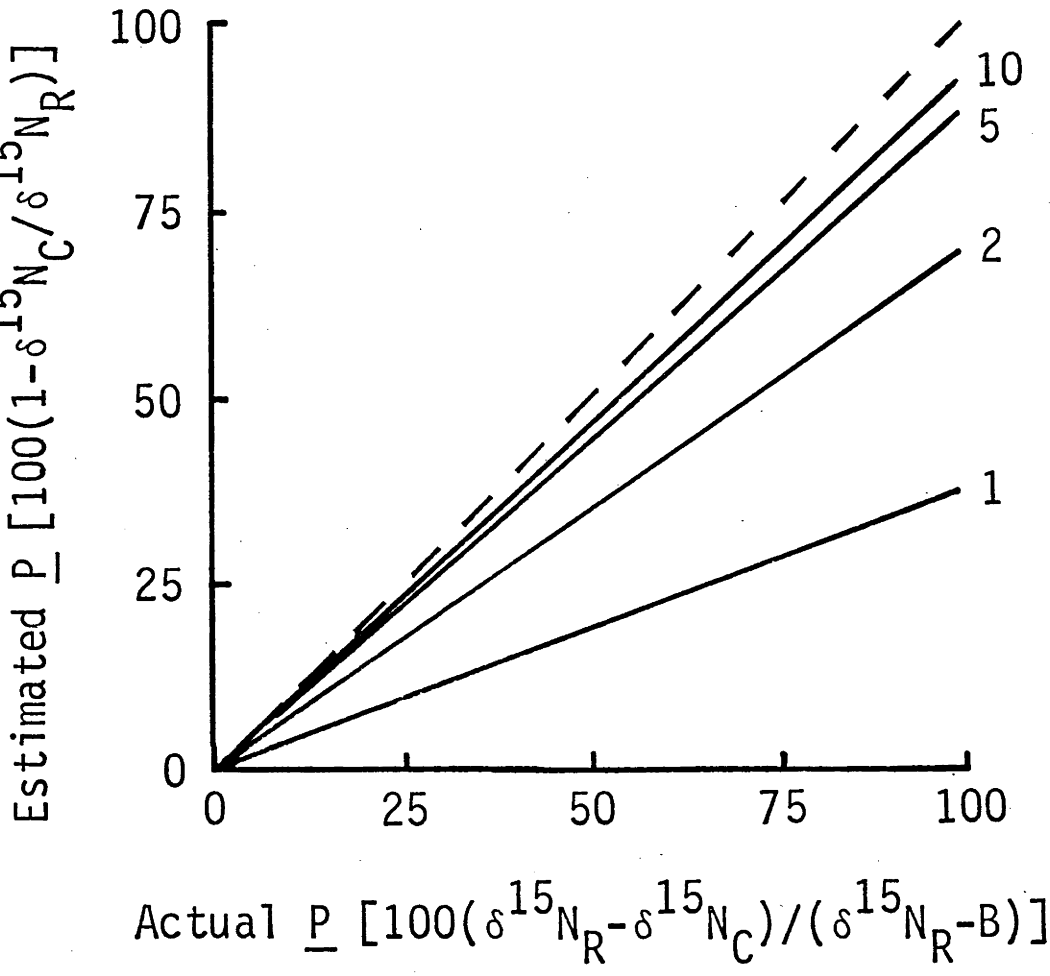


Figure 5.3. Effect of $\delta^{15}N$ of the reference plant ($\delta^{15}N_R$: values shown beside each line) on estimation of the proportion (P) of legume nitrogen fixed in relation to isotopic fractionation during N_2 fixation (B = 0.59‰, see Table 5.1) by subterranean clover (C).

CHAPTER 6

ISOTOPIC FRACTIONATION DURING REDUCTION OF NITRATE AND NITRITE BY PLANT EXTRACTS

6.1 Introduction

In the natural ^{15}N abundance and ^{15}N isotope dilution methods for estimating N_2 fixation by legumes, a reference plant is generally used to estimate the isotopic composition of N assimilated from the soil by the legume. Thus, it is assumed that there is no isotopic fractionation during N uptake from the soil or that it is the same for the legume and reference plant. Studies with established plants have generally shown that there is little or no isotopic fractionation during uptake of nitrate and it has been concluded that it has no significant effect on the field estimation of N_2 fixation by ^{15}N techniques (see section 2.4.4.2). However, Mariotti et al. (1982) measured considerable isotopic fractionation during uptake of nitrate-N by millet during the first week of growth, although it decreased with time (Fig. 2.1). They proposed that this initial effect was due to isotopic fractionation by the cytosolic nitrate reductase (NR) enzyme involved in the reduction of nitrate to nitrite within the plant since the NR activity was low initially and increased with time. This hypothesis remains untested. The nitrite produced by NR is then reduced to ammonia by the nitrite reductase (NiR) enzyme in chloroplasts before the N is assimilated into organic compounds (Fig. 6.1). Potentially, isotopic fractionation could occur during all assimilation processes.

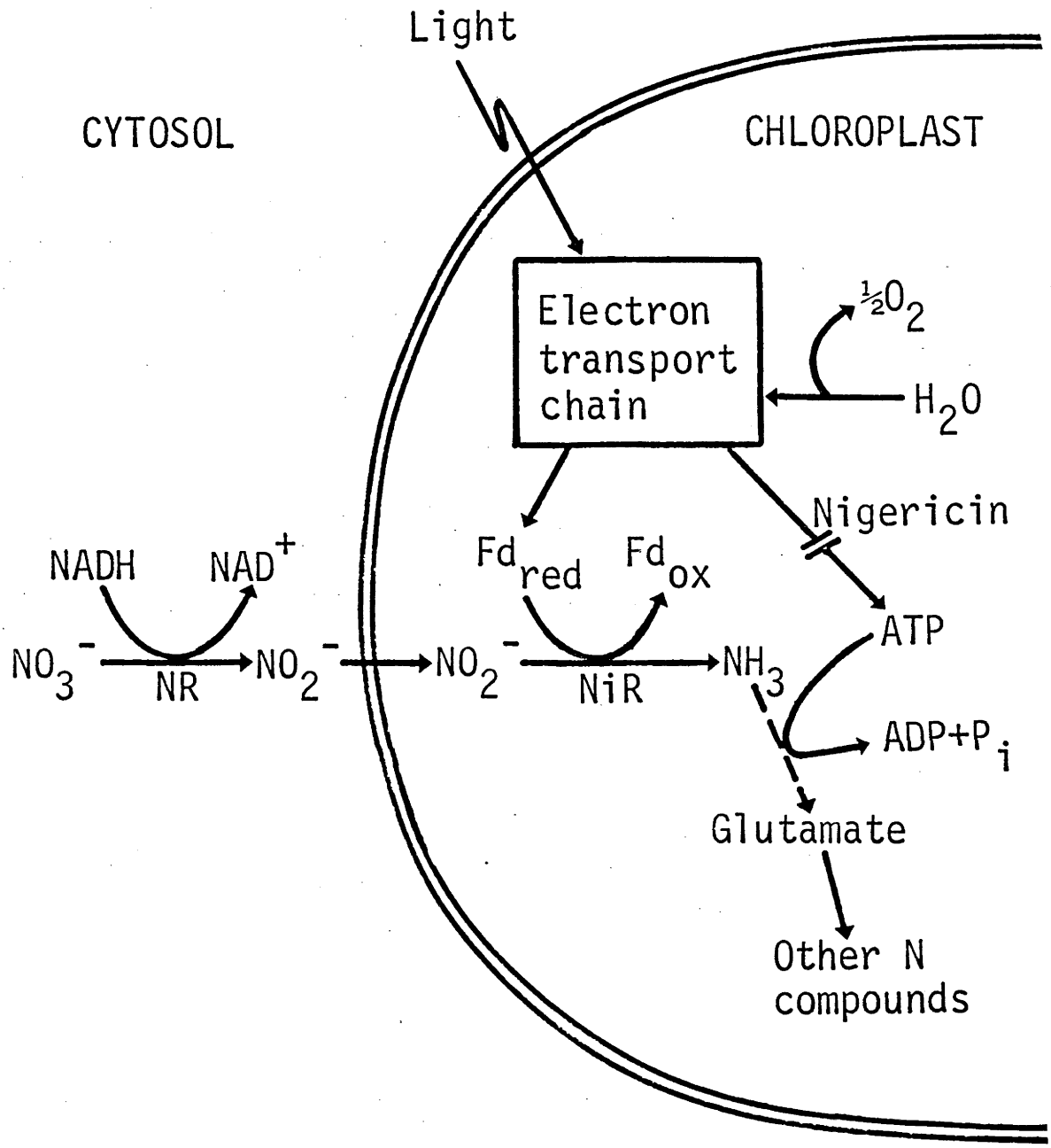


Figure 6.1. A simplified representation of nitrate metabolism in leaves of higher plants. Adapted from Silsbury and Ross (1978).

The purpose of this study was to determine the isotopic fractionation in each step during the reduction of nitrate to ammonia. Because of the difficulties involved in recovering nitrite formed from nitrate in an intact system, a reconstituted system consisting of the nitrate reducing system in a cytosolic and the nitrite reducing system in a chloroplastic fraction was used for the conversion of nitrate to ammonia. In addition, the reduction of nitrite to ammonia by NiR-alone was determined separately in a chloroplast-only system. When these systems were developed for optimal activity, they were scaled up for isotopic analysis.

6.2 Experimental

6.2.1. General procedures

6.2.1.1 Plant culture

Spinach (Spinacia oleracea L.) plants were grown hydroponically in a glasshouse. Hoaglands solution (Hewitt 1966) containing 11 mM NO_3^- was used as the growth medium.

6.2.1.2 Nitrite, ammonia, chlorophyll and protein assays

Nitrite was assayed by the sulphalinamide-(1-naphthyl) ethylenediamine.HCl method (Hewitt and Nicholas 1964; Miflin 1974). Ammonia was determined by the method of Chaney and Marbach (1962), as described by Bergersen (1980).

Chlorophyll (Chl) was assayed by the procedure of Arnon (1949). A 1:100 (v:v) mixture of chloroplasts and 80% acetone was centrifuged at 15,000 g for 5 min. The absorbance (A) of the supernatant at 652 nm was determined and the concentration of Chl calculated as follows:

$$Chl\ (\mu g\ ml^{-1}) = A_{652} \times 27.8 \times 100.$$

The protein content of the cytosolic extracts was measured by the method of Lowry et al. (1951) using bovine serum albumin as standard.

6.2.1.3 Separation of cytosolic extracts

Freshly harvested, fully-expanded leaves were deribbed and ground with a mortar and pestle using a 1:1 (w:v) ratio of leaves and grinding solution. The grinding solution (G) contained 5 mM dithiothreitol to prevent oxidation of sulphydral groups, 0.1% bovine serum albumin to inactivate proteases, and 0.2% polyvinyl-pyrrolidone 40 to inactivate polyphenols, in medium C. Medium C consisted of 0.3M sorbitol, an asmoticum, 2 mM disodium EDTA to chelate heavy metals, 1 mM $MgCl_2.6H_20$ and 1 mM $MnCl_2.4H_20$ to activate enzymes, 0.5 mM KH_2PO_4 for phosphorylation by chloroplasts, and 50 mM HEPES buffer adjusted to pH 7.6. The ground suspension was filtered through two layers of Miracloth and the filtrate was centrifuged at 21,000 g for 20 min to remove particulate matter. The supernatant was added to a Sephadex G-25 column (10 mm diameter x 120 mm long) and eluted with medium C, to remove small molecules, including nitrate.

6.2.1.4 Isolation of chloroplasts

Deribbed leaves were homogenized in a breaking medium A using a 1:10 (w:v) ratio, respectively. Medium A was identical to solution G, except that 50 mM MES buffer, pH 6.1, was used instead of HEPES as chloroplasts are more active under slightly acid conditions and the polyvinyl-pyrrolidone 40 was replaced with 2 mM isoascorbate

to maintain reducing conditions. The homogenate was filtered though two layers of cheesecloth and three layers of Miracloth, and the filtrate was centrifuged at 1,500 g for 75 sec. The pellet was carefully resuspended in a total volume of 35 ml medium C and centrifuged at 370 g for 60 sec. This pellet contained chloroplasts and was resuspended in 2 ml of medium C before use.

6.2.1.5 Nitrate reductase activity

NADH oxidation and nitrite production procedures were compared for assaying the NR activity (NRA) of the cytosolic extracts. The rate of NADH oxidation was measured spectrophotometrically at 340 nm in a 1 ml volume containing 1 mM dithiothreitol, 0.2 mM NADH, 40 mM KNO_3, 100 µl medium C and cytosolic extract (equivalent to about 200 µg protein). After 4 min, the reaction was stopped by adding 100 µl of 1M zinc acetate and the nitrite produced was analysed.

6.2.1.6 Nitrite reductase activity

Nitrite reductase activity (NiRA) of chloroplasts was assayed by measuring O_2 evolution in the light (1 mEinstein $m^{-2}s^{-1}$) polarographically with a Clark O_2 electrode. The chloroplasts (equivalent to about 100 µg Chl) were incubated at 25°C in a reaction mixture (3.3 ml) containing 10 mM DL-glyceraldehyde (to inhibit CO_2 fixation), catalase (180 U ml^{-1}; to remove any H_2O_2 produced), 2.2 mM $NaNO_2$ and medium C.

6.2.2 Development of a system for studying isotopic fractionation during reduction of nitrate and nitrite

6.2.2.1 Preliminary studies of nitrite reduction

Preliminary studies on the requirements for optimum NiRA by chloroplasts-alone involved using the same procedure as described in section 6.2.1.6, except that 0.3 µM nigericin, to inhibit glutamate formation and enhance reduction of Fd by uncoupling ATP formation (Fig. 6.1), was included in the reaction mixture. After a fixed incubation period, the reaction was stopped by adding 380 µl of 1M zinc acetate and the mixture was centrifuged at 21,000 g for 20 min to precipitate the proteins. Ammonia in the supernatant was collected by diffusion and analysed.

6.2.2.2 Isotopic fractionation studies of nitrite reduction

On the basis of the preliminary studies the following scaled up reaction was used. Chloroplasts containing about 4.5 mg chlorophyll were incubated in a glass conical flask (250 ml) with 0.3 µM nigericin, 10 mM DL-glyceraldehyde, catalase (180 U ml^{-1}), 5 mM $NaNO_2$ and medium C in a final volume of 60 ml. This reaction mixture was shaken in a 25°C waterbath and illuminated at 500 µEinstein $m^{-2}s^{-1}$. After 30 min, the reaction was stopped by the addition of 10 ml of 1M zinc acetate and the mixture was centrifuged at 21,000 g for 20 min. Ammonia in the supernatant from two such reaction mixtures was recovered by diffusion and analysed for ^{15}N.

6.2.2.3 Preliminary studies of nitrate reduction

Preliminary studies on the requirements for optimum activity of a reconstituted system of cytosolic extract (containing NR) and

chloroplasts (containing NiR) involved using the same procedure as outlined in section 6.2.2.1 except that the reaction mixture also contained 0.2 mM NADH and cytosolic extract (about 3 mg protein), and the $NaNO_2$ was replaced with 40 mM KNO_3. The volumes of cytosolic extract and chloroplast suspension used were adjusted to achieve a known NRA to NiRA ratio. Oxygen evolution (an estimate of NiRA), and NO_2^- and NH_3 production were measured.

6.2.2.4 Time course studies of nitrate reduction

Before isotopic fractionation studies were conducted, the temporal patterns of production of NO_2^- and NH_3 by the reconstituted system were examined in a scaled up version of the preliminary studies. In these studies, cytosolic extract containing about 50 mg protein and chloroplasts (sufficient to achieve a NRA to NiRA ratio of 1:5) were incubated in a glass conical flask (250 ml) with 0.3 µM nigericin, 10 mM DL-glyceraldehyde, 1 mM NADH, catalase (180 U ml^{-1}), 40 mM KNO_3 and medium C in a final volume of 80 ml. Assay conditions were the same as described in section 6.2.2.2. Aliquots (9 ml) of reaction mixture were removed at 2, 5, 10, 15, 20 and 30 min after commencing incubation and the reaction was stopped by addition of 1.5 ml of 1M zinc acetate, and the mixture centrifuged at 21,000 g for 30 min. Nitrite and ammonia in the supernatant were recovered and analysed.

6.2.2.5 Isotopic fractionation studies on nitrate reduction

The procedure used in these studies was the same as that outlined in section 6.2.2.4 except that the final volume of the reaction

mixture was 20 ml and that additions of NADH and NO_3^-, in amounts equivalent to those in the initial reaction mixture, were made at 10 and 20 min, and at 15 min respectively after the start of the experiments. The reaction mixture was incubated for a total of 30 min and the reaction was stopped with 15 ml of 1M zinc acetate, and the mixture centrifuged at 21,000 g for 30 min. A control treatment, in which the zinc acetate was added to the reaction mixture prior to the initial NO_3^- addition, was also used to measure background NH_3 and its isotopic composition. Ammonia in the supernatant from the reaction mixture was recovered by diffusion for ^{15}N analysis.

6.2.2.6 Recovery of ammonia by diffusion

The procedure of Conway (1939) adapted by Bergersen (1980) was used to recover NH_3 from the reaction mixtures. Samples of the reaction mixture were adjusted to pH 11.5 with 10M NaOH in a conical flask sealed with a rubber stopper. Filter paper impregnated with 0.2 mmol H_2SO_4 was suspended above the mixture by a stainless steel wire inserted into the rubber stopper. The assemblies were held at 20°C until all NH_3 was recovered. This method was chosen to minimise hydrolysis of amides present in the plant extracts (Bergersen 1980).

In the preliminary studies involving a small volume of reaction mixture (about 3 ml), 25 ml flasks were used and the diffusion period was 18 hr. However, in the ^{15}N studies the procedure was scaled up to provides sufficient NH_3 for ^{15}N analysis; about 100 ml of reaction mixture was used in 1l flasks and the diffusion period was 5 days.

After incubation, the filter paper was removed and transferred to a vial containing 1 ml of distilled H_2O. After mixing, an aliquot

was taken for NH_3 analysis and where appropriate, the remainder was analysed for ^{15}N. The ^{15}N concentrations of the NO_3^- and NO_2^- substrates were determined after reduction to NH_3 by distillation (see section 3.3.2.4).

6.3 Results

6.3.1 Properties of the system used

6.3.1.1 Activity of the individual enzymes

There was no significant difference between the estimates of NRA in the cytosolic extracts by the NADH oxidation or the NO_2^- production methods (Table 6.1). Thus, the simpler NADH oxidation procedure was chosen as the main method for assaying NRA. Nitrate reductase activity was essentially the same whether KNO_3 was added at 2.5, 10, 20 and 40 mM and therefore 40 mM was used in all subsequent assays because of the desire to maintain a high substrate concentration in ^{15}N studies (see section 3.4).

During the reduction of NO_2^- in chloroplasts in the light, rates of O_2 evolution were 1.3-1.7 times higher than those for NH_3 production (Table 6.2). When the uncoupler, nigericin, was omitted from the reaction mixture, the NiRA was lowered (Table 6.2).

6.3.1.2 Activity of the reconstituted system

The ratios of net O_2 evolved to NH_3 produced in the reconstituted system containing cytosolic extract and chloroplasts were relatively constant and similar to those found for chloroplasts-alone (compare Tables 6.3 and 6.2). Net values for O_2 evolution were required because O_2 consumption was recorded in all cases with the combined NR/NiR system where there was no increase in NH_3

Table 6.1. Nitrate reductase activity of a cytosolic extract of spinach as determined by NADH oxidation or nitrite production. All rates were calculated from differences between treatments with and without added KNO_3 (40 mM final concentration) over a 4 min period.

Experiment number	Replicate number	net NO_2^- production (nmol mg protein^{-1} min^{-1})	
		NADH oxidation	NO_2^- production
1	1	14.7	15.4
	2	15.4	16.7
2	1	11.4	11.1
	2	12.2	12.4
3	1	15.7	17.1
	2	16.5	16.5

Table 6.2. Nitrite reductase activity by illuminated spinach chloroplasts as determined by oxygen evolution or ammonia production. Chloroplasts were incubated for 8 min with 10 mM DL-glyceraldehyde, 0.3 μM nigericin, catalase (180 U ml^{-1}) and 2.3 mM NO_2^-, unless stated otherwise.

Treatment	Products measured [nmol (mg Chl)$^{-1}$ min^{-1}]		
	O_2	NH_3	O_2/NH_3[1] (S.E.)
Minus NO_2^-	13	107	
Complete	863 (850)[1]	700 (593)[1]	1.43 (0.04)
Minus nigericin	506 (493)[1]	447 (340)[1]	1.47 (0.15)
S.E.D.	50	37	0.15

[1]Corrected for the minus NO_2^- treatment.

Table 6.3. Production of nitrite, oxygen and ammonia from nitrate by a reconstituted system of cytosolic extract and isolated chloroplasts from spinach leaves as influenced by the ratios of nitrate reductase activity (NRA) to nitrite reductase activity (NiRA). All measurements were made after a 15 min photoperiod, unless stated otherwise.

NRA:NiRA[1]	Treatment	Total products measured (nmol)			
		NO_2^-	O_2	NH_3	O_2/NH_3[4]
(a) 1:2.3					
	Complete: mean	195	233 (443)[2]	335 (285)[2]	1.57
	: S.E.	18	17	15	0.11
	Complete: (dark)	380	-60	60	
	+ Triton (0.03%)	380	-190	70	
	- enzyme	0	-230	30	
(b) 1:4.7					
	Complete	90	610 (850)[3]	750 (660)[3]	1.3
	Complete (27 min)	60	870 (1310)	1130 (1040)	1.3
	Complete (dark)	380	- 70	70	
	- chloroplasts	400	- 90	70	
	- enzyme	0	-180	40	
	- NADH	0	-160	50	
	- NO_3^-	0	-240	90	

[1]Ratio of moles of NO_3^- to NO_2^- reduced. To achieve these NRA:NiRA ratios, the amounts of cytosolic protein and chlorophyll were 3.42 mg and 133 µg in (a) and 3.48 mg and 262 µg in (b), respectively (total volume was 3.3 ml). The NRAs of the cytosolic extracts were 11 and 12 nmol NO_2^- (mg protein)$^{-1}$ min^{-1} for (a) and (b), respectively. The NiRAs of the chloroplasts were 670 and 760 nmol NH_3 (mg chlorophyll)$^{-1}$ min^{-1} for (a) and (b), respectively.

[2]Corrected for the average of the -enzyme and + Triton treatments.

[3]Corrected for the -NO_3^- treatment (a measured value of -440 nmol O_2 was used for the -NO_3^- treatment for 27 min).

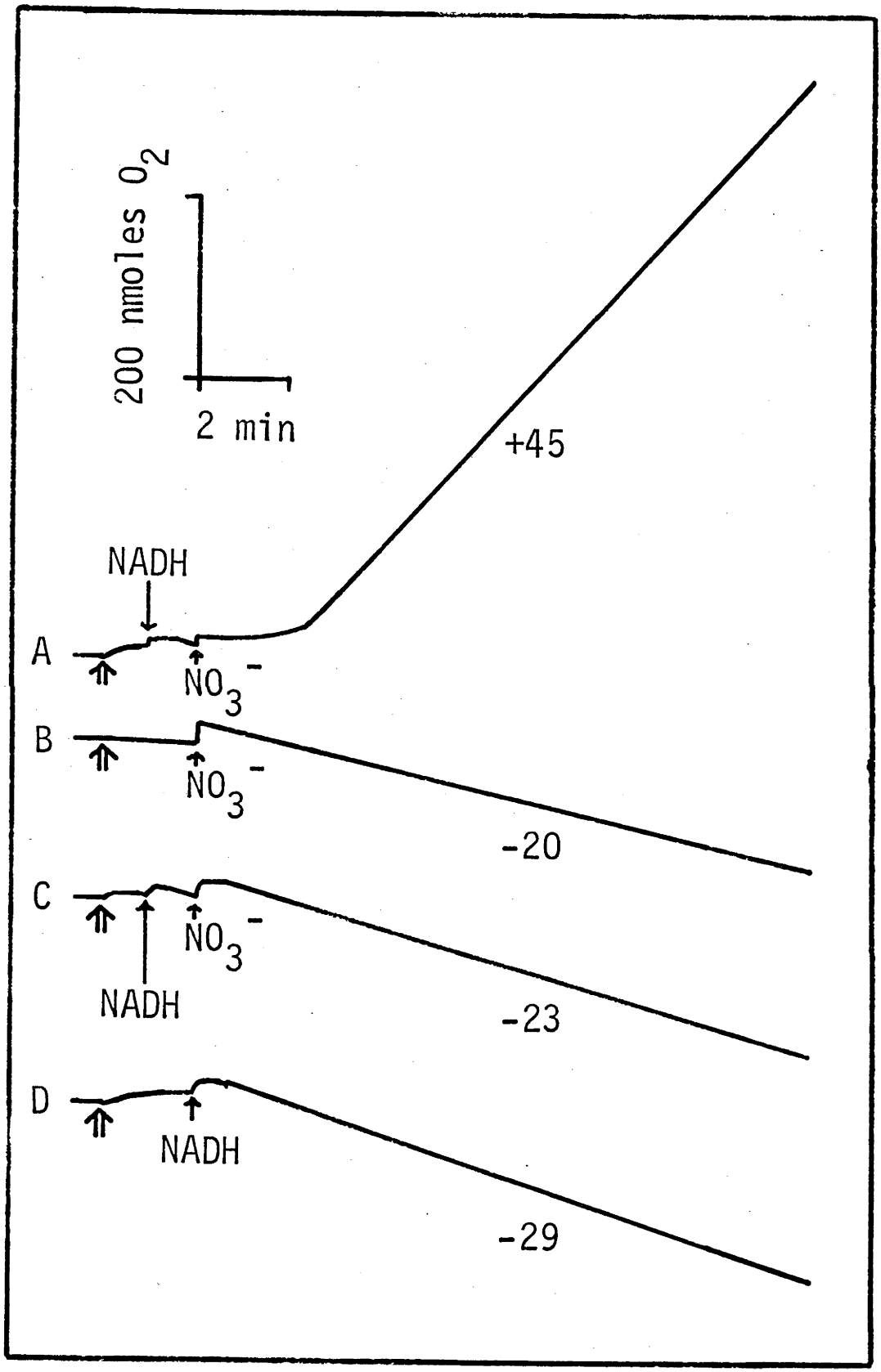
[4]Using corrected values.

production (compare Table 6.3 and Fig. 6.2). Rates of O_2 production or consumption were linear for the 15 min measurement period (Fig. 6.2).

In order to establish that the reconstituted system functioned as shown in Fig. 6.1 and that the system required a substrate before NH_3 was produced, the effects of varying the constituents of the system were examined. No NO_2^- was produced (Table 6.3) and O_2 was consumed (Fig. 6.2) when either the NR, NADH or NO_3^- were omitted from the system, although a minor amount of NH_3 was formed. Maximum amounts of NO_2^- were produced when the chloroplasts were omitted, when Triton X-100 was used to disrupt the chloroplasts or when dark conditions were maintained. An increase in NH_3 production and O_2 evolution (over that of the background) required NR (cytosolic fraction), NiR (chloroplasts), NADH, NO_3^- and light. Under these conditions, when the NRA:NiRA ratio was 1:2.3, the amount of NH_3 formed was 1.3-1.7 times that of the NO_2^- (Table 6.3a). When the ratio of activities was increased to 1:4.7, the amount of NH_3 formed was 7.3-17.3 times that of the NO_2^- (Table 6.3b). The combined production of NO_2^- and NH_3 over the 15 min period was close to that estimated from the NRA (e.g. 78-95%, Table 6.3a).

The NO_3^--dependent O_2 evolution increased markedly as the NRA:NiRA (i.e. cytosolic:chloroplastic fraction) ratio increased from 1:1 to 1:3 (Fig. 6.3). The effect of NADH concentration on NO_3^--dependent O_2 evolution was also examined using a NRA:NiRA ratio of 1:4. Values obtained for net O_2 evolution were 7.9, 9.8, 9.2, 9.8 mmol O_2 (mg protein)$^{-1}$ min^{-1} at NADH concentrations of 0.1, 0.5, 1.0 and 2.0 mM respectively. Thus, maximum activity by the reconstituted system was obtained at about 0.5 mM NADH.

Figure 6.2. Pattern of oxygen evolution or consumption from a reconstituted system of cytosolic extract and chloroplasts (see Table 3 for details). A, complete; B, minus NADH; C, minus cytosolic extract; D, minus NO_3^-. Double-tailed arrows show the time of illumination. Single-tailed arrows denote times of addition of NO_3^- or NADH. Values beside the curves represent rates of oxygen production in nmol min^{-1}.


200 nmoles O_2
2 min
+45
NADH
A
NO_3^-
B
NO_3^-
-20
C
NO_3^-
NADH
-23
D
NADH
-29

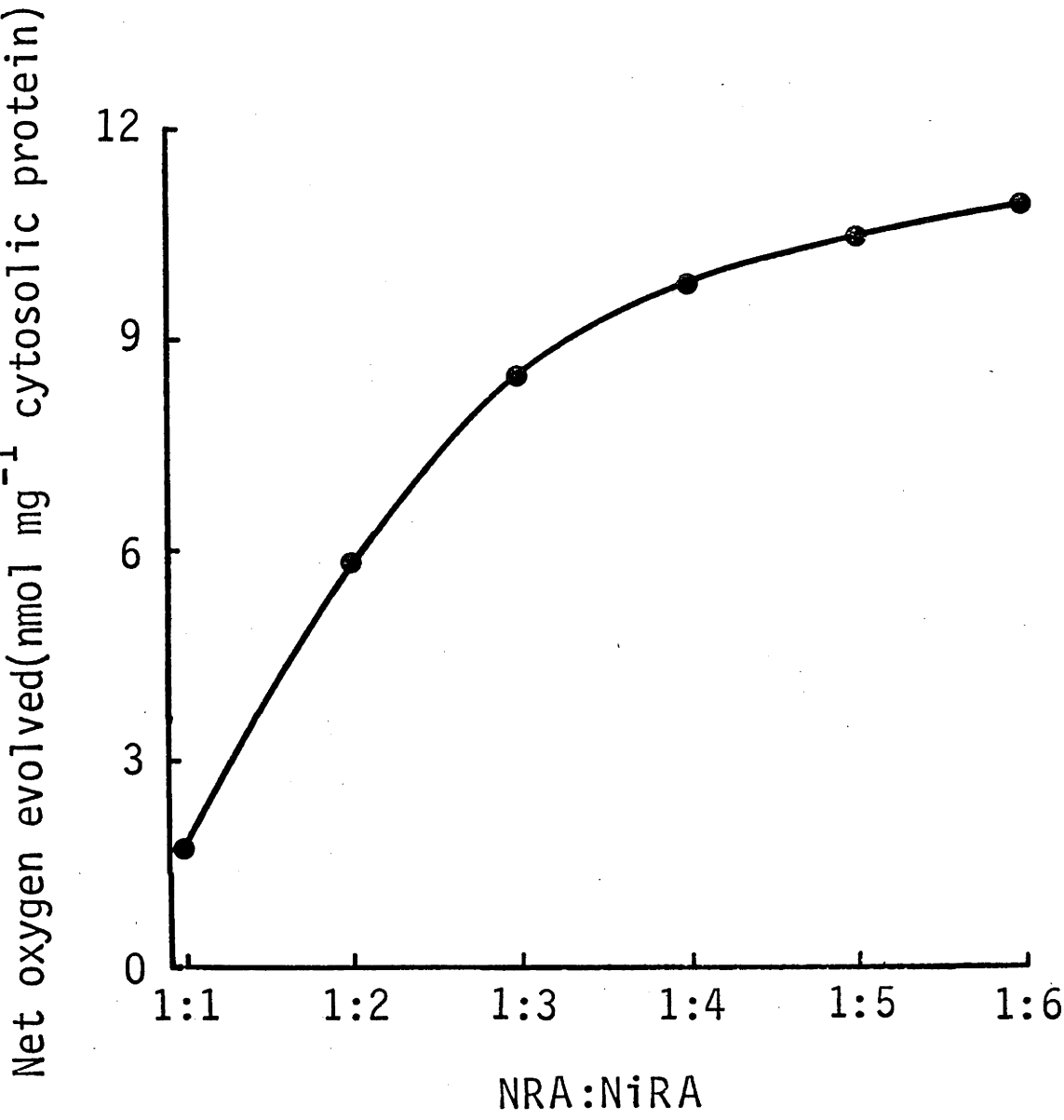


Figure 6.3. Effect of the ratio of nitrate reductase activity (NRA) to nitrite reductase activity (NiRA) on net nitrate-dependent oxygen evolution from a reconstituted system of cytosolic extract and chloroplasts from spinach leaves. Cytosolic extract (3.3 mg protein) with a NRA of 29.4 nmol NO_2^- min^{-1} was used for all measurements.

Time course studies on large volume, reconstituted systems showed that there was a lag in the production of NH_3 for 10-15 min after addition of NO_3^-, but linear rates were subsequently obtained (Fig. 6.4). There appeared to be an increase in the amount of NO_2^- produced throughout the measurement period, but levels were small compared with the amounts of NH_3 produced (7-10% at 30 min).

6.3.2 Isotopic fractionation

A study on the changes in $\delta^{15}N$ of NH_3 evolved from an $(NH_4)_2SO_4$ solution (adjusted to pH 11.5) in the large (1 l) diffusion flasks showed that diffusion was incomplete after two days (a $\delta^{15}N$ of -3.01‰ with respect to the solution being measured). However, by days 4 and 6, the $\delta^{15}N$ of the diffused NH_3 was the same as that of the original solution. Thus, a five day period was chosen for diffusion of the samples for ^{15}N analysis. Four replicates of the $(NH_4)_2SO_4$ solution in medium C and zinc acetate were also diffused and analysed for ^{15}N. The $\delta^{15}N$ of the NH_3 produced (2.99 ± 0.14‰) was not significantly different from that of the original solution (2.89 ± 0.02‰).

The $\delta^{15}N$ of NH_3 produced by the reconstituted system was significantly ($P < 0.01$) lower than that of the substrate NO_3^- (Table 6.4). Thus, isotopic fractionation (-8.85‰) was obtained (Table 6.4). Depletion of the substrate NO_3^- during the reaction was small (< 5%) and therefore unlikely to have affected this estimate. In the reconstituted system, the NH_3 produced from the control (i.e. inactivated enzymes) was relatively large (ca. 20%) compared with that of the active system (Table 6.4). If the estimate of isotopic

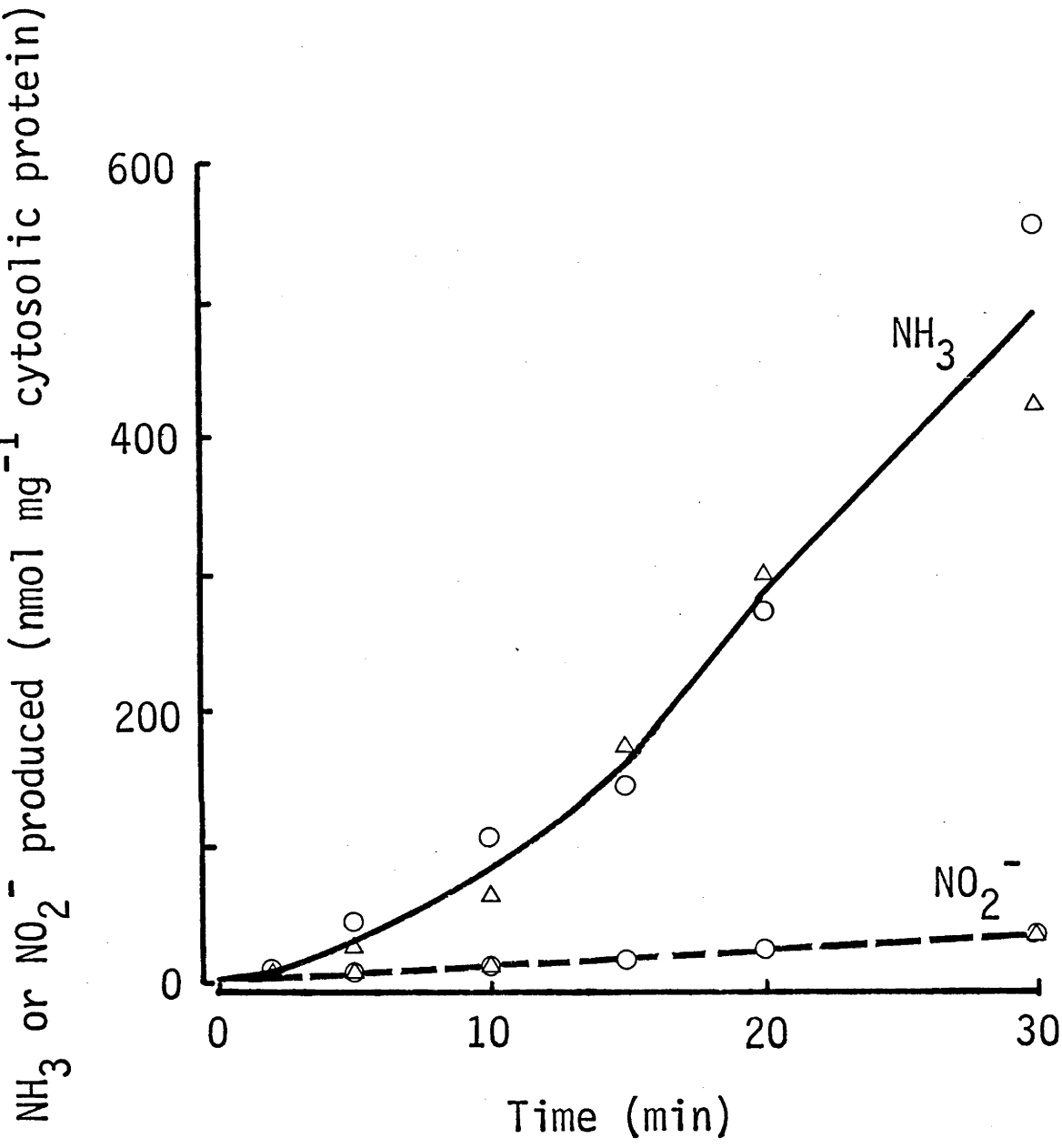


Figure 6.4. Nitrite and ammonia production from nitrate by a reconstituted system of cytosolic extract and chloroplasts from spinach leaves. The ratio of nitrate reductase activity to nitrite reductase activity was 1:5. Values are given for two separate experiments (○, △).

Table 6.4. Isotopic composition and rate of production of ammonia from the reduction of nitrite by chloroplasts (total volume = 120 ml) and from the reduction of nitrate by a reconstituted system of cytosolic extract and chloroplasts (the ratio of NR activity to NiR activity was 1:5; total volume = 60 ml). Reactions were stopped after 30 min by addition of zinc acetate, or prior to substrate addition with the control treatments. Each value is the mean of four replicate experiments except the control values which are for one replicate.

	Total NH_3 formed [μmol (mg Chl)$^{-1}$]	$\delta^{15}N$ (‰)[1]	$\Delta\delta^{15}N$ (‰) uncorrected[2]	$\Delta\delta^{15}N$ (‰) corrected[3]
Chloroplast system				
Substrate (NO_2^-)		-96.62	-0.42 (3.03)[4]	
Product (NH_3)	6.03	-97.04		
Control (NH_3; inactive system)	0.21	-		
Reconstituted system				
Substrate (NO_3^-)		-19.75	-8.85 (0.58)[4]	-14.89
Product (NH_3)	8.91 (1.14)[5]	-28.60		
Control (NH_3; inactive system)	1.70 (0.22)[5]	- 2.98		

[1] With respect to atmospheric N_2.

[2] $\Delta\delta^{15}N = \delta^{15}N_{substrate} - \delta^{15}N_{product}$.

[3] Calculated as $\Delta\delta^{15}N = \frac{(AB)_{product} - (AB)_{control}}{B_{product} - B_{control}} - A_{substrate}$

where A = $\delta^{15}N$, and B = μmol NH_3 formed.

[4] S.E.

[5] μmol NH_3 (mg cytosolic protein)$^{-1}$.

fractionation is adjusted for this 'background' NH_3, a value of -14.89‰ is obtained (Table 6.4).

There was no significant difference between the $\delta^{15}N$ of the NH_3 produced by the chloroplast system and that of the substrate NO_2^- (Table 6.4), indicating that there was no isotopic fractionation during NO_2^- reduction. Although there was significant depletion in the NO_2^- substrate during the assay (ca. 10%), this will have had little effect on the estimate of isotopic fractionation (i.e. -0.42 and -0.44‰ using equations 38 and 39, Chapter 3, respectively). In the NiR system, the amount of NH_3 produced from the control was insufficient for ^{15}N analysis (Table 6.4). If the $\delta^{15}N$ of the NH_3-N produced by the control was the same as that for the control in the reconstituted system, the estimate of isotopic fractionation for NiR, adjusted for the control would be about -4.4‰. This value is also unlikely to be significantly different from zero. Thus, isotopic fractionation in the chloroplast system was negligible compared with that of the reconstituted system.

6.4 Discussion

6.4.1 Properties of the system used

In the chloroplast studies, the ratios obtained for NH_3 production relative to net oxygen evolution (where CO_2 fixation was inhibited) ranged from 1:1.3 to 1:1.7; these are in agreement with the operation of a photosynthetically-coupled nitrite reduction, viz.,

$$H_2O + HNO_2 \xrightarrow[NiR]{h\nu} NH_3 + \frac{3}{2} O_2.$$

The rates of NiRA varied from 19 to 45 μmol (mg Chl)$^{-1}$ hr^{-1} and are similar to the short-term rates recorded by Plaut et al. (1977). These rates were relatively low when nigericin was omitted from the reaction mixture, presumably due to a decrease in the formation of reduced ferridoxin and therefore a decrease in the rate of NiRA (see Fig. 6.1).

The results from the reconstituted system of cytosolic extract and chloroplasts support the scheme for NO_3^- metabolism outlined in Fig. 6.1. Thus, in this system, NO_2^- was only produced by NR when NO_3^- (the substrate) and NADH (an electron donor) were added. The subsequent production of NH_3 from NO_2^- was dependent on light and active chloroplasts, demonstrating that NiR is located within the chloroplasts (Magalhaes et al. 1974; Miflin 1974). These assays revealed that near maximum NH_3 production required the NiRA to be at least three times the NRA. In the leaves of most plants the NRA:NiRA ratio generally ranges from 1:5 to 1:20 (Beevers and Hageman 1980), indicating that NR activity could be a limiting step in NO_3^- assimilation.

6.4.2 Isotopic fractionation

Isotopic fractionation was measured during the reduction of NO_3^- to NH_3 and this could have occurred at several steps: 1) reduction of NO_3^- to NO_2^- by NR, 2) diffusion of NO_2^- into the chloroplasts, 3) reduction of NO_2^- to NH_3 by NiR, and 4) diffusion of NH_3 out of the chloroplasts. However, steps 2, 3 and 4 were also present in the studies on the reduction of NO_2^- to NH_3 in which no isotopic fractionation was measured. This indicates that the observed fractionation was associated with the reduction of NO_3^- to NO_2^- by NR.

The isotopic fractionation factor (calculated as $\Delta\delta^{15}N$, Table 6.4) of -15‰ (adjusted for background N) is similar to the value (-18‰) estimated for isotopic fractionation during absorption of NO_3^- by 3 days-old seedlings of millet (adjusted for seed N) by Mariotti et al. (1982; Fig. 2.1). These authors proposed that this was due to isotopic fractionation associated with NR, at a stage when NR was limiting the assimilation of NO_3^-. However, they measured little change in the amount of N in the seedlings relative to that in the seed (for similar changes, see Fig. 5.1) and therefore the error associated with the isotopic fractionation factor must have been large. Also, their observed isotopic fractionation could have been associated with remobilization of seed N.

If isotopic fractionation during any one process (e.g. NO_3^- reduction or remobilization of seed N) is to be expressed in terms of differences in ^{15}N concentration between total plant N and substrate N, some loss of N from the plant must occur. Loss of plant N is only likely to occur by direct excretion from roots (e.g. Virtenan et al. 1937) or by gaseous losses from above-ground parts (e.g. Wetselaar and Farquhar 1980). Direct excretion of nitrogenous compounds from intact roots of growing plants can occur, but the conditions for this are very specific and it is not considered important under normal growing conditions (Butler et al. 1959). The subject of gaseous losses of N from above-ground parts of plants was reviewed by Wetselaar and Farquhar (1980) and Farquhar et al. (1983) and they concluded that losses of NH_3 from the stomata may be significant. However, if significant losses occur, they appear to be confined to the period late in plant growth, between anthesis and maturity, and associated with breakdown of proteins in senescing leaves (Thimann

1980). If diffusion of NH_3 is the step limiting loss of NH_3 from the stomata, it can be calculated that the $\delta^{15}N$ of NH_3 being lost from the plant will be -18‰ relative to the source (Farquhar et al. 1983). Unfortunately, the magnitude of NH_3 loss from plants is unknown, although it is thought to be small (< 3 nmol $m^{-2}s^{-1}$; G.D. Farquhar, pers. comm.).

The reduction of NO_3^- to NH_3 involves a series of linked reactions (Fig. 6.1) from which there are no branch points and no sites from which N loss from the plant can occur. Therefore, any isotopic fractionation during NO_3^- reduction is unlikely to be expressed as a difference in ^{15}N concentration between the plant and the NO_3^- supplied in the medium.

In growing plants, isotopic fractionation by NR could result in residual NO_3^-, in the immediate vicinity of NR, having a higher instantaneous ^{15}N concentration than that of the reduced N, but this will tend to equilibrate with time because of mass balance. Even if the reduced-N in plants was depleted in ^{15}N relative to the residual NO_3^--N in plants, this effect would rarely be measured in the analyses of the isotopic composition of total plant N. All plant N analyses carried out in the experiments described in this thesis involved a salicylic acid modification of the Kjeldahl digestion to ensure that all NO_3^- was reduced to ammonium (see section 3.2.2.1). When the conventional Kjeldahl digestion was used, about 50% of the NO_3^--N was converted to ammonium (Table 3.1).

Mariotti et al. (1980) found that the isotopic composition of total N in a range of plant species was the same as that of the NO_3^- in the medium in which they were grown. Similarly, in pot experiment 1, the isotopic composition of ryegrass N was the same as that of N

mineralized from the soils in which it was grown (Fig. 4.3). These findings suggest that N losses from the plants were negligible and therefore any isotopic fractionation that may have been associated with the assimilation of substrate N was not expressed as differences in ^{15}N concentration between plant N and substrate N.

In many highly-productive pasture soils in Australasia, plant production is limited by low soil N availability (Brougham et al. 1978; Ball 1979; Steele and Shannon 1982). This is reflected by low levels of NO_3^--N in plant material (Ledgard and Saunders 1982). The limiting step in assimilation of soil N by plants would then be the initial uptake. In these conditions, any absorbed N would tend to be assimilated as rapidly as it is absorbed, thereby reducing the observed isotopic fractionation. Thus, under field conditions isotopic fractionation during assimilation of soil N may not be expressed, but even if it is, the effect is unlikely to be reflected in differences between the isotopic composition of total plant N and soil N.

CHAPTER 7

EFFECT OF LOW RATES OF NITROGEN ADDITION ON THE FIXATION AND TOTAL ACCUMULATION OF NITROGEN BY LEGUMES

7.1 Introduction

A basic assumption in the use of the ^{15}N isotope dilution method for measuring N_2 fixation by legumes is that the application of small amounts of ^{15}N-labelled N to the soil will have no effect on N_2 fixation or the N accumulated by the legumes. Most studies on the effect of added N on N_2 fixation by established legumes have involved much higher rates of N addition than those used in ^{15}N isotope dilution studies and have generally shown a reduction in the amount of N_2 fixed (see section 2.4.3.1). In pot experiments, Vallis et al. (1967) found that 0.75 ppm N [about 6 kg N ha^{-1} applied as KNO_3 and $(NH_4)_2SO_4$] caused an increase in the N accumulated by Townsville lucerne and Rhodes grass of about 11%, while Haystead and Lowe (1977) found no effect of 2 kg N ha^{-1} [as $(NH_4)_2SO_4$] on the N accumulated by white clover and ryegrass. In both bases, the effect on N_2 fixation was not examined.

The aim of the present study was to examine the effects of low levels of N addition, typical of those used in ^{15}N isotope dilution studies, on the fixation and total accumulation of N by legumes. This involved preliminary acetylene reduction experiments with subterranean clover grown alone in sand, a pot experiment with subterranean clover grown with annual ryegrass in soil and a field experiment with

subterranean clover and lucerne grown in association with either annual ryegrass or phalaris.

7.2 Experimental

7.2.1 Acetylene reduction experiments

Assay vessels were constructed so that regular non-destructive acetylene reduction assays could be carried out (see section 3.2.4 and Plate 3.1). They were filled with washed sand and eight pregerminated subterranean clover (cv. Woogenellup) seeds were planted. Commercial peat inoculant (Nodulated type C, Agricultural Laboratories, Sefton, N.S.W.) was washed into the surface of the sand. Distilled water and a N-free nutrient solution (see section 5.2) were applied daily. The vessels were kept in a glasshouse with temperatures ranging between about 12 and 28°C.

After six weeks growth, the following N treatments were imposed:

Experiment 1 0, 5 and 10 kg N ha^{-1} x 7 replicates

2 0, 2 and 4 kg N ha^{-1} x 7 replicates

3 0 and 1 kg N ha^{-1} x 10 replicates.

Sodium nitrate was used as the source of N and this was applied in solution to the surface of the sand. To ensure that there was no differential effect of sodium (Na), NaCl was applied so that all treatments received the same amount of Na. Following the imposition of treatments, the vessels were placed over plastic saucers (see Plate 3.1) and the collected leachate was returned to the surface of the sand.

Before measuring acetylene reduction, the vessels were allowed to drain freely for 18 hours with no water or nutrient solution being

applied. Acetylene reduction assays were carried out several days prior to imposition of N treatments, and at 2, 5 and 10 days after application of NO_3^-. The time chosen for the assays was about midday, to obtain near maximum rates of nitrogenase activity. The acetylene reduction method (described in section 3.3.4) involved analysis of gas samples collected at 20 and 80 minutes after injection of acetylene and propylene.

7.2.2 Pot experiment 3

This experiment was conducted mainly to assess plant recovery of added and soil N (for details, see section 10.3.1) but it also enabled examination of the effect of low levels of addition of NO_3^- on the fixation and total accumulation of N by subterranean clover grown in association with annual ryegrass.

Thirty-four days after sowing, plants were trimmed to 20 mm and treatments were imposed. These were, a control (no added N) and two rates of ^{15}N-labelled $NaNO_3$ (0.46 and 1.54 mg N pot^{-1}, equivalent to 0.3 and 1.0 kg N ha^{-1}). The N was applied to the soil surface in solution (10 ml pot^{-1}) and washed in with 50 ml distilled water. There were 24 replicates of each N rate and 12 replicates of the control. Twenty-one days after trimming, plants (shoots + roots) were harvested, separated into clover and grass components and analysed for total N and ^{15}N. Fixation of N_2 was determined by natural ^{15}N abundance for the control and ^{15}N isotope dilution where NO_3^- was added.

7.2.3 Field experiment

The effect of addition of 1 kg N ha^{-1} on the fixation and total accumulation of N by subterranean clover and lucerne grown in

association with either annual ryegrass or phalaris was also studied in a field experiment (for details, see section 9.2.1).

All plots were trimmed to 20 mm and KNO_3 (1 kg N ha^{-1}, 66 atoms % ^{15}N) was applied to the soil surface of the ^{15}N isotope dilution treatments and washed in with water equivalent to 2 mm precipitation. Twenty-five days later, all plots were harvested (shoots only) and plant material was separated into legume and grass components for analysis of total N and ^{15}N. Fixation of N_2 was determined as for pot experiment 3.

7.3 Results

7.3.1 Acetylene reduction experiments

In experiment 1, the application of $NaNO_3$ at 5 kg N ha^{-1} caused a decrease (31%) in the rate of ethylene (C_2H_4) production 2 days after NO_3^- application, but there was no significant effect on days 5 and 10 (Table 7.1). However, the effect of 10 kg N ha^{-1} was still evident on day 5 (45% decrease) but had disappeared by day 10 (Table 7.1).

There was no significant effect of 2 or 4 kg N ha^{-1} on C_2H_4 production in experiment 2, even on day 2 (Table 7.1). The rates of C_2H_4 production were markedly higher than in experiment 1 and the errors were correspondingly higher.

In experiment 3, rates of C_2H_4 production were much lower than those in experiment 2, and application of 1 kg N ha^{-1} caused a small (23%) but significant ($P < 0.01$) reduction in the rate of C_2H_4 production on day 2; there was no significant effect by day 5 (Table 7.1).

Table 7.1. Effect of $NaNO_3$ on acetylene-dependent ethylene production (μmoles hr^{-1} $vessel^{-1}$) by subterranean clover grown alone in sand in a glasshouse. Each value in experiments 1 and 2 is the mean of seven replicates and in experiment 3, the mean of ten replicates.

	$NaNO_3$ addition (kg N ha^{-1})	Prior to N application	Days after N application		
			2	5	10
Experiment 1	0	5.76	6.44	5.32	8.51
	5	5.64	4.43	5.01	8.21
	10	5.92	2.10	2.93	7.80
	S.E.D.	0.28	0.29	0.32	0.64
Experiment 2	0	7.49	8.47	9.29	-
	2	7.75	8.89	9.30	-
	4	7.86	9.11	8.44	-
	S.E.D.	0.43	0.70	0.80	
Experiment 3	0	4.72	4.53	5.67	-
	1	4.60	3.51	5.16	-
	S.E.D.	0.22	0.23	0.32	

7.3.2 Pot experiment 3

There was no significant effect of rates of application of $NaNO_3$ equivalent to 0.3 and 1.0 kg N ha^{-1} on the amounts of N assimilated by clover or ryegrass during the 21 day period of observation (Table 7.2). Also, there was no significant difference in the P values estimated for the control and added NO_3^- treatments (Table 7.2).

7.3.3 Field experiment

There was no significant effect of KNO_3 at 1 kg N ha^{-1} on the assimilation of N by the legumes or grasses during a 25 day period of growth in the field (Table 7.3). Similarly, there was no significant difference in the estimates of P for lucerne, determined by the natural abundance (no added N) or isotope dilution (+ 1 kg N ha^{-1}) methods, whether grown with ryegrass or phalaris. However, different estimates of P were obtained by these two methods for clover; values obtained by the natural abundance method were higher than those obtained with isotope dilution, particularly when phalaris was the associated grass.

7.4 Discussion

The acetylene reduction experiments revealed that the addition of $NaNO_3$ at rates as low as 1 kg N ha^{-1} can produce a temporary diminution in nitrogenase activity of subterranean clover. They also suggest that the detrimental effect of N addition on N_2 fixation can be influenced by environmental conditions, being less where conditions were favourable for high rates of N_2 fixation. In experiment 2, there was a longer daylength and higher average temperature (by 5°C) than for experiments 1 and 3 and this favoured higher rates of growth and nitrogenase activity. Bethlenfalvay and Phillips (1978) found that

Table 7.2. Effect of $NaNO_3$ on the nitrogen accumulated by subterranean clover and annual ryegrass and on the proportion (P) of clover nitrogen fixed. The plants were grown together in pots in a glasshouse. Each value of the 0 N treatment is the mean of 12 replicates and of the 0.3 and 1.0 N treatments, the mean of 24 replicates.

N rate ($kg\ ha^{-1}$)	Clover N ($mg\ pot^{-1}$)	Grass N ($mg\ pot^{-1}$)	P (%)
0	20.5	59.6	95.6
0.3	21.3	60.9	91.6
1.0	22.9	60.6	90.8
S.E.D.a[1]	1.8	2.3	4.0
b	1.4	2.0	2.1

[1] a refers to 0 vs. 0.3 or 1.0; b refers to 0.3 vs. 1.0.

Table 7.3. Effect of the addition of $K^{15}NO_3$ at 1 kg N ha^{-1} on the nitrogen accumulated by shoots of legume or grasses and on the estimate of the proportion (P) of legume nitrogen fixed in various legume, grass associations 25 days after NO_3^- addition to field plots. Each value is the mean of six replicates.

	Clover, ryegrass	Clover, phalaris	Lucerne, ryegrass	Lucerne, phalaris
Legume N (g m^{-2}):				
control	4.58	6.77	2.00	1.81
+ 1 kg N ha^{-1}	4.26	6.09	1.70	1.44
S.E.D.	0.61	0.58	0.40	0.22
Grass N (g m^{-2}):				
control	2.86	1.56	2.96	2.55
+ 1 kg N ha^{-1}	2.92	1.95	2.74	2.55
S.E.D.	0.38	0.21	0.37	0.35
P (%):				
control	85.2	85.7	81.0	63.9
+ 1 kg N ha^{-1}	70.4	49.9	88.1	70.0
S.E.D.	4.8	7.4	5.9	6.6

higher rates of photosynthesis in peas counteracted the inhibition of N_2 fixation by added N and they attributed this to a stronger sink for fixed N_2.

The significance of the effect of added N on the total amount of N_2 fixed may be influenced by the measurement period; at low rates of addition of NO_3^- the diminished nitrogenase activity was temporary. Thus, although there may have been an initial decrease in N_2 fixation due to added NO_3^- in the pot and field experiments, any effect would have been small relative to the remaining growth period when there was no such effect. In the pot and field experiments, grasses growing in association with the legumes would compete with the legumes for uptake of added NO_3^-, thereby reducing further any effect of the added NO_3^- on N_2 fixation. Walker et al. (1956) found that the assimilation of added N by white clover was small in the presence of ryegrass. However, in the pot and field experiments reported here there was no effect of added NO_3^- on N accumulation by either legumes or grasses, illustrating that rates of N application up to 1 kg N ha^{-1} are sufficiently small to have no significant effect on the assimilation of N from the soil or atmosphere. The fact that different estimates of P for clover were obtained in the field when natural abundance and isotope dilution methods were used while similar estimates were found in the pot experiment is thought to be a consequence of the methods and is discussed in detail in Chapter 9.

These studies showed that 1 kg N ha^{-1} can be used safely in ^{15}N isotope dilution experiments without affecting N_2 fixation. Care should be taken if higher rates than this are to be used, particularly during periods of slow growth; the effects of N_2 fixation and total N accumulation should be examined first.

When a long measurement period is used, it may be necessary to apply ^{15}N-labelled compound at more than 1 kg N ha^{-1} so that the plant-available soil N has a sufficiently high ^{15}N label throughout the period. However, instead of using one high rate of N addition, several applications of N at a low rate could be used during the measurement period to avoid any possible effects on N_2 fixation or total N accumulation (e.g. Vallis et al. 1967; Goh et al. 1978). This would also result in less variation in ^{15}N concentration of the plant-available soil N with time compared with using one large initial application.

CHAPTER 8

SOURCE OF NITROGEN ASSIMILATED BY THE NON-LEGUMINOUS REFERENCE PLANT

8.1 Introduction

With ^{15}N methods for measuring N_2 fixation by legumes, a non-N_2-fixing reference plant is commonly used to estimate the isotopic composition of the portion of N which is assimilated by the legume from the soil. It is apparent that these methods will give incorrect estimates of N_2 fixation if the reference plant has N_2-fixing bacteria associated with it or if fixed N_2 is transferred directly to the reference plant from the associated legume.

In mixed legume/grass pastures, grasses are commonly used as reference plants because they are not infected by N_2-fixing rhizobia, as may occur if uninoculated legumes are used as reference plants. However, grasses may promote N_2 fixation by free-living bacteria growing in their rhizosphere by excreting compounds which serve as energy sources for the bacteria (reviewed by van Berkum and Bohlool 1980). The N_2 fixed by these bacteria can then become a significant source of N for the grass (Day et al. 1975a; Greenland 1977).

In mixed legume/grass pastures, N can also be supplied directly to the grass by transfer of fixed N_2 from the associated legumes. However, this process is believed to be relatively slow and is not considered to be important in the short term (see section 2.4.3.5).

The aim of this study was to investigate whether these two processes would supply sufficient N to an associated grass to

significantly affect the determination of N_2 fixation by legumes when the natural ^{15}N abundance or ^{15}N isotope dilution methods are used.

8.2 Experimental

8.2.1 N_2 fixation associated with ryegrass

Two variations of an acetylene reduction technique (an in situ method and an isolated core method; section 3.3.4) were used to determine whether any nitrogenase activity was associated with annual ryegrass growing at the field experimental site (section 3.2.1). The study involved four replicates of three treatments; bare soil (no plants present), ryegrass alone and ryegrass with subterranean clover.

Gas samples were collected 30, 100, 160 and 370 minutes after injection of C_2H_2 and C_3H_6 with the in situ method and after 25, 85, 145 and 355 minutes for the isolated core technique and analysed for C_2H_2, C_2H_4 and C_3H_6 (section 3.3.4).

8.2.2 Transfer of nitrogen from legumes to ryegrass

Two ^{15}N-labelling techniques were used to determine whether N was transferred directly from legumes to ryegrass growing together in pots in the glasshouse or in field plots.

8.2.2.1 ^{15}N-labelling of legume

A new method for measuring transfer of N from the legume to an associated grass was developed and tested. With this method, legume plants were labelled by foliar absorption of solutions containing ^{15}N-labelled compounds at the start of a growth period. At the end of this period the legume and grass plants were harvested and

analysed for total N and ^{15}N. Unlabelled legume and grass plants were also used as controls. Thus, this method estimates the transfer to an associated grass of the N present in the legume at the start of a growth period. The initial ^{15}N-labelled legume N may be measured on separate plants or determined by assuming that it is equivalent to that in the legume at the end of the growth period plus that transferred to the grass. The proportion of legume N transferred to the grass (T_L) is then calculated from

$$T_L = \frac{N_g (G_\ell - G_u)}{N_l(L_\ell - L_u) + N_g(G_\ell - G_u)} \qquad (41)$$

where Ng and N_l are the amounts of N assimilated by the grass and legume respectively, G_ℓ and G_u are the atoms % ^{15}N of the labelled and unlabelled grass plants and L_ℓ and L_u are the atoms % ^{15}N of the labelled and unlabelled legume plants, respectively (for derivation of this equation see Appendix 1). The amount of N transferred from the legume to the grass is obtained by multiplying T_L by the amount of N assimilated by the legume (N_l).

8.2.2.1.1 Foliar absorption of ^{15}N

A preliminary study was made to test the feasibility of using the ^{15}N-labelling technique for measuring N transfer from legumes to associated grasses. The effect of different methods of labelling the legume on the ^{15}N concentration of the various plant parts was studied.

Subterranean clover (cv. Woogenellup) seeds were sown into twelve pots containing washed river gravel. The pots were kept in a glasshouse and regularly given applications of distilled water and a N-free nutrient solution (as described in section 5.2). After six weeks growth, three replicates of the following four treatments were imposed for 48 hours:

1) three trifoliate leaves were immersed in 30 mM KNO_3 solution,
2) three petioles (trifoliate leaves removed) were immersed in 30 mM KNO_3 solution,
3) three trifoliate leaves were immersed in 15 mM $(NH_4)_2SO_4$ solution, and
4) an untreated control.

The N solutions (containing 95 atoms % ^{15}N) were contained in glass vials inside small plastic bags with self-sealing tops and the leaves (or petioles) were placed into these solutions. The plastic bags were sealed around the petioles of the plants to avoid contamination and to ensure that the leaves (or petioles) remained immersed in the N solution. These plastic bags were then taped to the sides of the pots.

After 48 hours immersion in the N solution, the clover plants were trimmed to 20 mm above the gravel surface. This ensured that there was no contamination of soil or remaining plant material by the leaves or petioles which had been immersed in ^{15}N-labelled solution. Plants were then left to grow for five days and harvested. Plant material was separated into shoots, roots and nodules and analysed for total N and ^{15}N.

8.2.2.1.2 Transfer of ^{15}N from legumes to grass in pots

This study was carried out in conjunction with pot experiment 4, section 10.3.2, and the soils and experimental procedure are given there. Subterranean clover and annual ryegrass seeds were sown together in six pots and later thinned to three and four plants per pot, respectively. Twenty-nine days after sowing, four trifoliate leaves from each of the three clover plants in three pots were immersed in a solution of 15 mM $(NH_4)_2SO_4$, containing 95 atoms % ^{15}N, as described above. Three days after the initial immersion in ^{15}N solution, the plant material was trimmed to 20 mm above the soil surface in all pots. Twenty-seven days later, ryegrass and clover plants were harvested, separated into shoots and roots and analysed for total N and ^{15}N.

8.2.2.1.3 Transfer of ^{15}N from legume to grass in the field

Subterranean clover/annual ryegrass and lucerne/annual ryegrass plots were used for this study and eight microplots were located within each of these (for details see section 3.2.1). Each microplot (150 mm diameter) contained one subterranean clover plant and four ryegrass plants or two lucerne plants and four ryegrass plants.

Three trifoliate leaves of the subterranean clover plants and one stem of the lucerne plants were immersed in solutions of 15 mM $(NH_4)_2SO_4$ containing 95 atoms % ^{15}N. The vials of ^{15}N solution were located in small plastic bags and these were taped to small metal pegs

to avoid spillage. Three days later, all plant material was trimmed to 20 mm height and the labelled shoots and ^{15}N solutions were carefully removed. At this stage, unlabelled control microplots and surrounding plots were also trimmed. Thirty six days later, each microplot was sampled by removing a soil core (150 mm diameter, 150 mm deep). Shoots were trimmed from the tops of the cores, roots were washed free of adhering soil, and the shoots and roots were separated into grass and clover components. Ryegrass shoots were also sampled at distances of 150 mm and 300 mm from the ^{15}N-labelled legumes. All plant material was analysed for total N and ^{15}N.

8.2.2.2 ^{15}N labelling of soil

The ^{15}N technique described by Vallis et al. (1967) was also used to measure the transfer of N from subterranean clover to annual ryegrass in the field. Potassium nitrate (66 atoms % ^{15}N) was applied at 1 kg N ha^{-1} to the soil surface in 700 x 450 mm microplots located within plots of ryegrass/clover and ryegrass alone (for details see section 9.2).

The microplots were trimmed to 20 mm height prior to ^{15}N addition and harvested 36 days later. Plant material was analysed for total N and ^{15}N and the proportion of grass N obtained from N_2 fixed by the associated clover (T_s) during the measurement period was estimated using the following expression (Vallis et al. 1967):

$$T_S = 1 - \frac{(\text{atoms \% } ^{15}N_G - B)}{(\text{atoms \% } ^{15}N_{GC} - B)} \qquad (42)$$

where G refers to grass grown alone and GC to grass grown with clover and B to the atoms % ^{15}N of clover grown with atmospheric N_2 as its only source of N. The amount of fixed N_2 transferred from the clover to the grass could then be obtained by multiplying T_s by the amount of N assimilated by the grass during the 36 day period.

8.3 Results

8.3.1 N_2 fixation association with ryegrass

When the isolated core method was used, no measurable C_2H_4 production was observed from the bare soil or ryegrass site during the first 85 minutes after C_2H_2 injection. When longer periods of incubation were used, small but negligible levels of C_2H_4 production were observed (Table 8.1). Ethylene production from cores containing subterranean clover was high, although the rates declined progressively after 85 minutes (Table 8.1).

When the in situ method was used, considerably higher levels of C_2H_4 were produced from the bare soil and ryegrass treatments than were found with the isolated core technique (Table 8.1). There was no significant difference in C_2H_4 production between these two treaments. Data for three replicates of the ryegrass treatment were similar to that for the bare soil treatment. However, one replicate had rates of C_2H_4 production up to six times higher than the other replicates. There was little difference between replicate measurements of the yield of shoots and roots of ryegrass in each of the cylinders. Also, there was no difference in the amount of dead roots (a potential source of carbon) present in the soil in each cylinder containing ryegrass. High rates of C_2H_4 production were obtained with the in situ method where subterranean clover was present; they were linear

Table 8.1. Acetylene-dependent ethylene production (μmoles m^{-2}) by bare soil, ryegrass and ryegrass + subterranean clover using a) isolated soil cores and b) an in situ method. Each value is the mean of four replicates.

a) Isolated cores	Minutes afer C_2H_2 injection			
	25	85	145	355
Bare soil	-	-	0.69	0.57
Ryegrass	-	-	0.40	0.80
S.E.D.[1]	-	-	0.20	0.26
Ryegrass + clover	68	282	399	672
(S.E.)	(51)	(198)	(290)	(516)
b) In situ	Minutes after C_2H_2 injection			
	30	100	160	370
Bare soil	3.2	5.1	9.1	25.2
Ryegrass	3.6	13.7	28.7	104.9
S.E.D.[1]	0.3	4.5	11.0	38.8
Ryegrass + clover	71	253	606	1437
(S.E.)	(35)	(89)	(282)	(525)
Soil temperature at 10 mm depth (°C)	14	17	18	16

[1] S.E.D. for comparison between bare soil and ryegrass only.

with time throughout the measurement period and were similar to the initial rates in the isolated core assays (Table 8.1). There was a large variation in the rates of C_2H_4 production from replicates of subterranean clover, although the variation was less than that obtained with the isolated core method (Table 8.1).

8.3.2 Transfer of nitrogen from legumes to ryegrass

8.3.2.1 Foliar absorption of ^{15}N

Immersion of either the trifoliate leaves or petioles of subterranean clover in ^{15}N-enriched solutions was an effective method of enriching all plant parts with ^{15}N (Table 8.2). The shoots were always more highly labelled than the roots or nodules whatever method or compound was used to label the plant (Table 8.2). When $(NH_4)_2SO_4$ was used as the N source, the shoots and nodules had a significantly higher ^{15}N enrichment than when KNO_3 was used (Table 8.2).

8.3.2.2 Transfer of ^{15}N from legume to grass in pots

The ^{15}N-labelling treatment had no significant effect on N accumulation by subterranean clover or ryegrass (Table 8.3). There was a larger error associated with the ^{15}N concentration of clover compared with ryegrass (Table 8.3) and this reflects the variability in ^{15}N concentration of the ^{15}N-enriched clover due to the labelling treatment. The ^{15}N concentration of ryegrass shoots was significantly higher in the ^{15}N-labelled treatment than in the control treatment. This result suggests that, on a whole plant basis, 2.2% of the clover N was transferred to the ryegrass during the 29 days after ^{15}N-labelling of the clover.

Table 8.2. Effect of form of added nitrogen and site of ^{15}N absorption on ^{15}N concentration in the shoots, roots and nodules of subterranean clover. Each value is the mean of three replicates.

Form of added N	Plant part used for ^{15}N absorption	Atoms % ^{15}N		
		shoots	roots	nodules
NO_3^-	petiole	1.2511	0.8698	0.5014
NO_3^-	leaf	1.2978	0.8667	0.4610
NH_4^+	leaf	2.5023	0.8775	0.5751
Control (no added N)		0.3666	0.3668	0.3665
S.E.D.		0.0328	0.0223	0.0231

Table 8.3. The amount and ^{15}N concentration of nitrogen in subterranean clover and annual ryegrass grown in pots in a glasshouse. Each value is the mean of three replicates.

		Clover			Ryegrass		
		shoots	roots	total	shoots	roots	total
mg N pot^{-1}:							
	Control	131.1	31.2	162.3	36.7	10.5	47.2
	Labelled	125.7	27.2	152.9	33.7	8.9	42.6
	S.E.D	8.1	3.3	9.2	2.5	1.3	3.1
Atoms % ^{15}N:							
	Control	0.3707	0.3784	0.3722	0.4024	0.4023	0.4024
	Labelled	0.5115	0.4313	0.4973	0.4132	0.4098	0.4125
	S.E.D.	0.0113	0.0164	0.0165	0.0018	0.0031	0.0033

8.3.2.3 Transfer of ^{15}N from legume to grass in the field

As in the pot experiment, the ^{15}N concentrations of the shoots and roots of clover and lucerne were significantly greater in the ^{15}N-labelled treatment than in the control treatment (Table 8.4). However, the labelling of the clover had no significant effect on the ^{15}N concentration of the ryegrass (Table 8.4), indicating that there was no transfer of N from either legume to the associated ryegrass plants during the 36 day period.

8.3.3 ^{15}N labelling of soil

The ^{15}N concentration in ryegrass grown alone was not significantly different from that in ryegrass grown in association with subterranean clover (Table 8.5), indicating that there was no detectable transfer of N from clover to ryegrass. The total N yield of ryegrass was considerably higher when it was grown alone than when grown with subterranean clover but the total plant recovery of added ^{15}N was similar for the two treatments (Table 8.5).

8.4 Discussion

8.4.1 N_2 fixation associated with ryegrass

When the natural ^{15}N abundance or ^{15}N isotope dilution methods are used to estimate N_2 fixation by legumes, it is assumed that there is no N_2 fixation associated with the reference plant. If this occurred, the ^{15}N concentration of the N assimilated from the soil by the legume will be underestimated and therefore $\underline{P}$ will be underestimated. The extent to which $\underline{P}$ is underestimated depends also on the value of $\underline{P}$, being greater at low $\underline{P}$ values (Fig. 8.1).

Table 8.4. The amount and ^{15}N concentration of nitrogen in legumes and grasses from field microplots 36 days after ^{15}N-labelling of legume shoots. The ^{15}N concentration of ryegrass, sampled 150 and 300 mm from the ^{15}N-labelled legumes, was also measured. Each value is the mean of four replicates.

	Within microplot				Outside microplot	
	Legume		Grass		Grass shoots	
	shoots	roots	shoots	roots	150 mm	300 mm
mg N microplot^{-1}						
Clover,ryegrass	88	22	55	14		
Lucerne,ryegrass	45	13	59	15		
Atoms % ^{15}N (Clover,ryegrass)						
Control	0.3668	0.3673	0.3682	0.3683		
Labelled	0.3976	0.3881	0.3685	0.3688	0.3682	0.3685
S.E.D.	0.0054	0.0069	0.0003	0.0004		
Atoms % ^{15}N (Lucerne,ryegrass)						
Control	0.3670	0.3674	0.3683	0.3682		
Labelled	0.4717	0.4451	0.3685	0.3687	0.3683	0.3684
S.E.D.	0.0073	0.0082	0.0002	0.0006		

Table 8.5. Effect of subterranean clover on the yield and isotopic composition of nitrogen in ryegrass shoots 36 days after ^{15}N application. Each value is the mean of six replicates.

	Atoms % ^{15}N in ryegrass	N yield (g m^{-2})	^{15}N excess yield[1] (mg m^{-2})
Ryegrass alone	0.658	3.81	11.11
Mixture: ryegrass	0.784	1.53	6.40
: clover		4.09	4.86
S.E.D.	0.068		

[1] Calculated as g N m^{-2} x (atoms % ^{15}N-0.366) x 10.

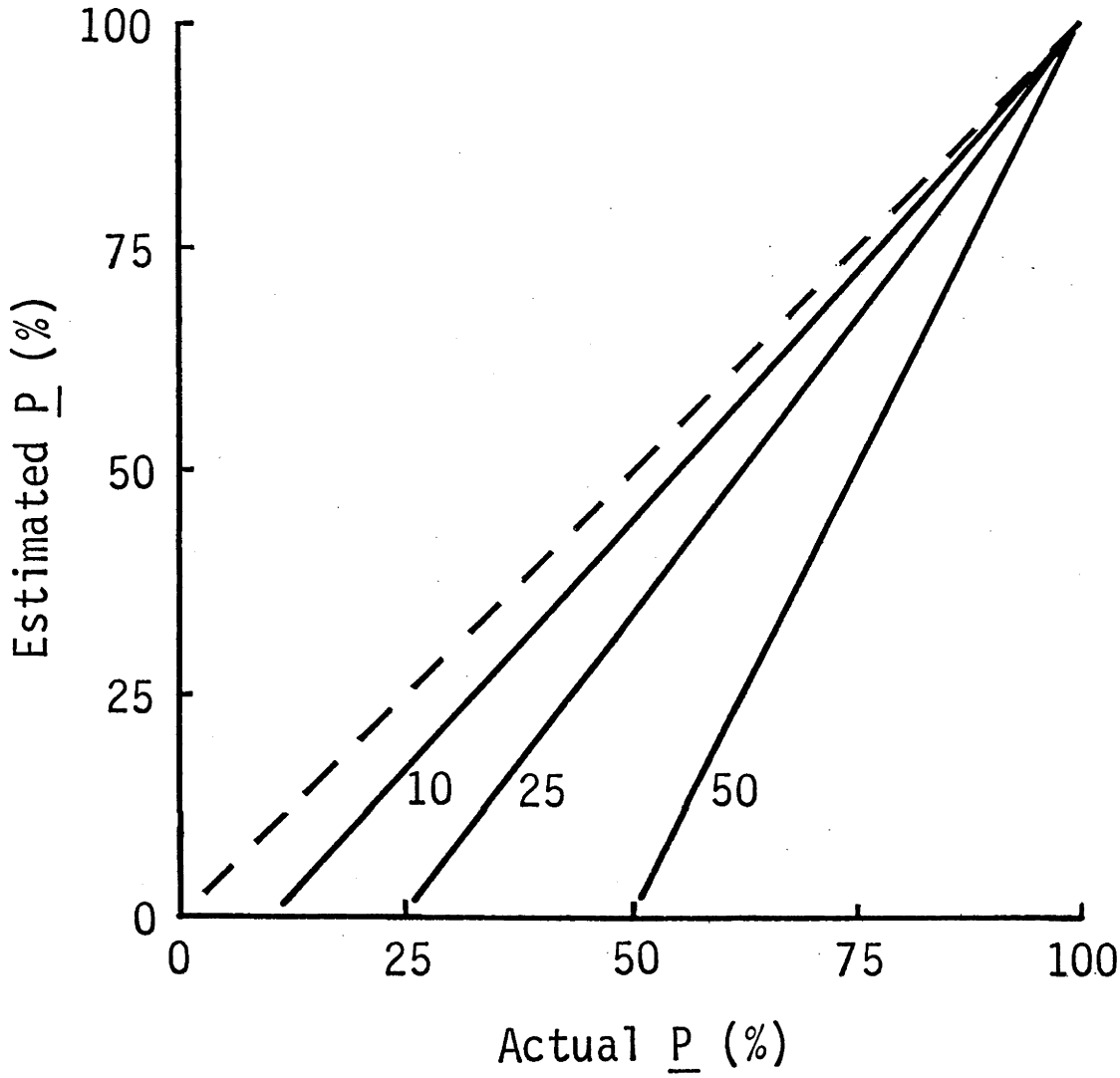


Figure 8.1. Effect of the level (in %) of N_2 fixation associated with the reference plant on the estimation of the proportion (P) of legume nitrogen fixed. This relationship is independent of the ^{15}N concentration of the reference plant. The broken line indicates where estimated P = actual P.

Two techniques were used to examine N_2 fixation associated with ryegrass in the field studies on legume N_2 fixation. There was a marked difference in the C_2H_2-dependent C_2H_4 production between the in situ and soil core techniques for bare soil and ryegrass treatments, but not for the subterranean clover treatment. This may be due to the free-living N_2-fixing bacteria being exposed to higher oxygen concentrations in the soil core technique due to soil disturbance and the relatively small volume of each core. Inhibition of N_2 fixation occurs at high oxygen concentrations (van Berkum and Bohlool 1980) and N_2-fixing bacteria in the bare soil and ryegrass treatments are likely to have had poor oxygen protection mechanisms (Neyra and Dobereiner 1977). This contrasts with the well developed oxygen protection mechanism of N_2-fixing bacteria enclosed in clover root nodules, which is unlikely to be affected by soil disruption (Robson and Postgate 1980). Thus the in situ technique probably gives a more realistic measurement of N_2 fixation by free-living bacteria in the field.

Rates of C_2H_4 production in bare soil and ryegrass treatments using the in situ technique were low. If the conventional C_2H_2:N_2 ratio of 3:1 (Hardy et al. 1973) is used, they extrapolate to 0.31-0.85 g N fixed ha^{-1} $hour^{-1}$. This is similar to values reported for other temperate grasses (Steyn and Delwiche 1970; Nelson et al. 1976; Thompson et al. 1984). However, in this study there was no difference between the bare soil and ryegrass treatments. Nitrogen fixed by free-living bacteria that are not directly associated with ryegrass will eventually enter the inorganic N pool in the soil and become equally available for uptake by both grass and clover. Thus, although this might tend to reduce the ^{15}N concentration of the

plant-available soil N it would not affect the estimate of P by clover with the natural ^{15}N abundance or ^{15}N isotope dilution methods. Furthermore, C_2H_4 production from the bare soil and ryegrass treatments was minimal (< 8%) compared with that from the subterranean clover treatment and even if all of the C_2H_4 produced from the ryegrass treatment was associated with ryegrass it would have had a negligible effect on the estimate of P by these methods.

8.4.2 Nitrogen transfer from legumes to ryegrass

In the measurement of N_2 fixation by legumes using the natural ^{15}N abundance or ^{15}N isotope dilution methods, transfer of fixed N_2 from the legume to the reference plant would also result in an underestimate of the ^{15}N concentration of the soil N assimilated by the legume. This would result in P being underestimated. The extent to which P is underestimated increases 1) as the amount of N transfer increases, 2) as the amount of N_2 fixed by the legume increases relative to the amount of N in the grass, and 3) as P approaches 50-70% (Fig. 8.2). Thus, when P nears 0 or 100%, transfer of fixed N_2 from the legume to the grass will have little effect on the estimate of P.

A new technique for estimating the 'underground' transfer of N from a legume to an associated reference plant using ^{15}N-labelling of the legume was examined. These studies showed that a relatively short period of immersion of a few legume leaves or petioles in ^{15}N enriched solution was sufficient to give a significant ^{15}N enrichment in nodules and roots as well as the shoots. This technique is more direct than that of the soil ^{15}N-labelling technique of Vallis et al. (1967) in that it measures transfer of legume ^{15}N to the reference

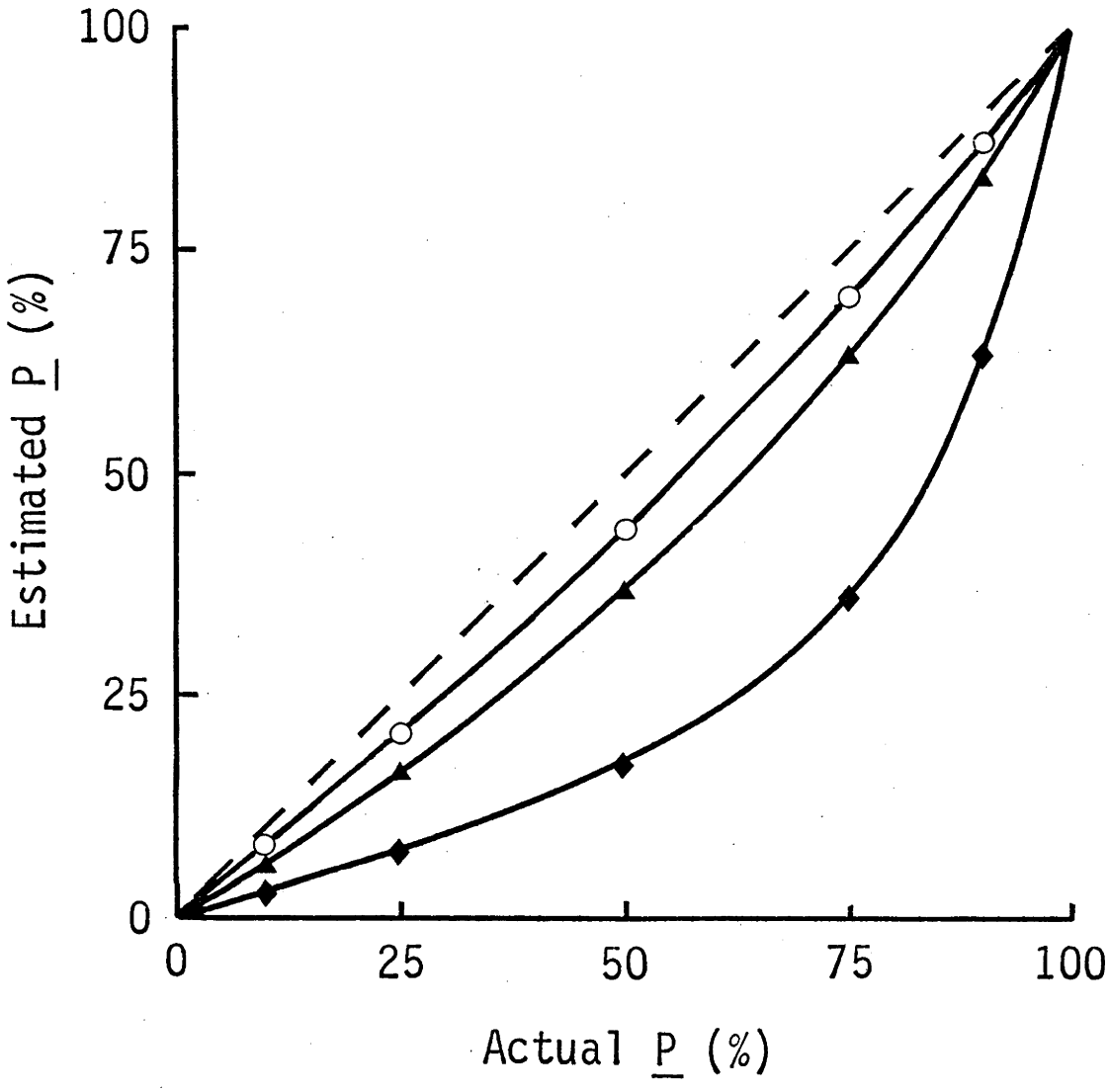


Figure 8.2. Effect of transfer of fixed N_2 (as % of total) from legume to reference plant on the estimate of the proportion (P) of legume nitrogen fixed. This relationship depends on the level of N_2 transfer and the nitrogen yield of the legume relative to the reference plant (ref.): ○——○ 20% transfer, legume N = ref. N; ▲——▲ 40% transfer, legume N = ref. N and 20% transfer, legume N = 2 x ref. N; ◆——◆ 40% transfer legume N = ref. N. The broken line indicates where estimated P = actual P.

plant. However, care must be taken in using this technique to avoid contamination of soil and plant material. In using this technique it is assumed that the ^{15}N absorbed by the legume labels all N compounds subject to N transfer. It is also assumed that, where equation 41 is used, the amount of ^{15}N-labelled legume N remaining in the soil at the end of the measurement period (e.g. that immobilized by soil microorganisms) is negligible. If this amount is significant, the denominator of equation 41 will be underestimated and therefore N transfer will be overestimated. This could be overcome by analysing separate ^{15}N-labelled legume plants at the start of the growth period and using these to estimate the amount of ^{15}N-labelled legume N (see section 8.2.2.1).

The legume ^{15}N-labelling technique measures the transfer to an associated grass, of the <u>total</u> legume N present at the time of labelling. In contrast, the soil ^{15}N-labelling technique measures the transfer to the grass, of N_2 <u>fixed</u> by the legume during the measurement period. There are two assumptions inherent in the soil ^{15}N-labelling technique: 1) the amount of ^{15}N assimilated is the same for the grass alone and grass/clover treatments, and 2) the level of plant-available soil N is the same in the grass alone and grass/clover plots. In applying this technique in the field, Vallis <u>et al</u>. (1977) found it to be invalid because the ^{15}N assimilated by the grass + clover exceeded that of the grass when grown alone. They suggested that the increased uptake of ^{15}N in the grass/clover plots may have been due to less immobilization of the added ^{15}N because of the higher N content of the plant residues, in the soil in these plots. Haystead and Lowe (1977) used the soil ^{15}N-labelling technique to estimate transfer of fixed N_2 from white clover to ryegrass in the

field and their data also showed that the grass alone and grass + clover differed in their amounts of ^{15}N assimilated. One would also expect that where the grass alone and grass/clover treatments have been established for a long period of time before assessing N transfer, the level of plant-available soil N would be higher in the grass/clover treatment due to the gradual increase in soil N from the death and decay of legume roots and nodules (Haystead and Marriott 1978). This could also invalidate estimates of N transfer by the soil ^{15}N-labelling technique.

When the soil ^{15}N-labelling technique was used in the field experiment reported here (Table 8.5), the amount of ^{15}N assimilated by the grass alone and grass/clover treatments was similar. There was also no significant transfer of fixed N_2 from clover to ryegrass during the 36 day measurement period. Similarly, with the legume ^{15}N-labelling technique, there was no detectable transfer of ^{15}N-labelled legume N to associated ryegrass plants. However, in the pot experiment there was a small amount of N transferred from the clover to ryegrass (2.2.%). This may have been due to a greater plant density and a large amount of roots restricted to a relatively small volume of soil in the pots. Thus, trimming the plants at the start of the measurement period may have induced a greater stress on the clover resulting in a greater transfer of N than in the field. Also, the greater density of grass roots in the pots may have increased the ability to absorb N released from the legume roots.

In the field study, the amount of ^{15}N absorbed by the leaves of the legume from the ^{15}N-labelled solution was less than in the pot study and therefore N transfer would be more difficult to detect. Nevertheless, it can be calculated (from Table 8.4) that a transfer of

legume N to the associated ryegrass of, for example, 10% would have increased the ^{15}N concentration of ryegrass (shoots and roots) to 0.3736 and 0.3760 atoms % ^{15}N for the clover and lucerne treatments, respectively, and these would be significantly higher than the ^{15}N concentrations of ryegrass in the control. Even a 2.2% transfer would have resulted in ryegrass containing 0.3691 and 0.3700 atoms % ^{15}N, respectively, and this would also have been detectable in the shoots.

The lack of significant transfer of N from the legumes to ryegrass in the field and of N_2 fixation associated with ryegrass (section 8.3.1) illustrate that in the field experiment on N_2 fixation by legumes (Chapter 9) the only source of N being assimilated by ryegrass was indigenous soil N or added ^{15}N-labelled N.

CHAPTER 9

ESTIMATION OF N_2 FIXATION IN FOUR LEGUME/GRASS ASSOCIATIONS IN THE FIELD

9.1 Introduction

In estimating N_2 fixation by legumes using ^{15}N methods it is necessary to have an accurate measure of the isotopic composition of the portion of N derived from the soil by a nodulated legume. This is commonly assessed by using a non-N_2-fixing reference plant growing in the same soil with or near to the legume.

In ^{15}N isotope dilution studies on established, mixed legume/grass pastures, the ^{15}N-labelled N cannot be incorporated into the soil without unacceptable soil disturbance and therefore it must be applied to the soil surface. In such a study it is considered important to match the legume and reference plant in their patterns of N uptake with soil depth (Phillips and Bennett 1978; Edmeades and Goh 1979). The extent to which the uptake patterns may affect the estimate of P has not been examined.

In established, mixed legume/grass pastures, reference plants cannot be introduced without disturbing the ecosystem and therefore it is necessary to use the indigenous non-N_2-fixing species as reference plants. This may mean choosing between several grass or weed species. There have been no published studies on the effect of using different indigenous reference plants on the estimate of N_2 fixation by pasture legumes.

In the present study, the natural ^{15}N abundance and ^{15}N isotope dilution methods were used to estimate N_2 fixation in four legume/grass associations. Pasture species which are known to vary in their depths of root penetration in the soil and which are of major importance in Australasian agriculture were chosen. The effect of differences in the timing and method of ^{15}N addition on the estimation of N_2 fixation was also studied using one of these associations. Two grasses and two weeds were used as reference plants.

9.2 Experimental

Subterranean clover or lucerne were grown in association with either annual ryegrass or phalaris (details on plant establishment are given in section 3.2.1).

On 23 September 1982 (day 0), all plots were trimmed to 20 mm height. Within each plot a ^{15}N treatment subplot, 700 x 450 mm, was made by inserting a galvanized sheet-iron frame into the soil to a depth of 100 mm and extending 20 mm above the soil surface (Plate 9.1). Nine of these subplots were established within each of the ryegrass/clover plots; all other plots had one subplot.

A solution of KNO_3 (66 atoms % ^{15}N) dried on to silica sand was applied to the subplots at 1 kg N ha^{-1} and washed in with 630 ml water (equivalent to 2 mm precipitation), except where indicated otherwise. For the treatments in the ryegrass/clover subplots there were several subtreatments.

1). The effect of method of ^{15}N application on the estimation of N_2 fixation was examined with:

Plate 9.1. A subterranean clover/phalaris microplot showing the plant species composition and galvanised sheet-metal frame used to inhibit lateral movement of $^{15}NO_3^-$.

(a) One application at day 0,

(b) One application at day 0 with 10 mm water, and

(c) Four applications of 0.25 kg N ha^{-1} at nine day intervals.

Harvests were made on (a) and (b) 10, 25 and 36 days after ^{15}N application with separate plots being used for each harvest; (c) was harvested on day 36 only.

2). The effect of time of ^{15}N application on the estimation of N_2 fixation in ryegrass/clover subplots was examined by:

(d) Applying ^{15}N on day 10 and harvesting plants on day 25, and

(e) Applying ^{15}N on day 25 and harvesting plants on day 36.

Harvests of untreated control (natural ^{15}N abundance) plots of ryegrass/clover were also made on days 0, 10, 25 and 36, using separate plots for each harvest.

The four legume/grass associations were used to examine the effect of the non-legume reference plant on the estimation of N_2 fixation. Harvests were made on days 0 and 25 for the control (natural ^{15}N abundance) treatments and day 25 for the ^{15}N enriched treatments. Additionally, from within two replicates of the ^{15}N-enriched subplots of ryegrass/lucerne and phalaris/lucerne, plant samples of volunteer subterranean clover, suckling clover, sorrel (Rumex acetosella L.) and chickweed [Stellaria media (L.) Vill.] were also collected and analysed for comparative purposes.

Replicates of all treatments were harvested by cutting an area 200x400 mm to ground level. For each replicate, the herbage was then mixed and a subsample taken for botanical and chemical analysis.

Samples containing roots were collected as follows from the control (natural ^{15}N abundance) and ^{15}N enriched treatment (1a

above) of the ryegrass/clover association. A metal corer (150 mm diameter) was used to collect two samples of plant and soil to a depth of 150 mm from each replicate of each treatment. Plant shoots were cut off at ground level and separated into grass and clover components. Roots were removed from the soil by sieving and washing and separated into grass and clover. All plant material was analysed for total N and ^{15}N.

Soil samples (0-50, 50-100, 100-150, 150-200 and 200-300 mm depth) were collected at the end of the experimental period from the ryegrass/clover treatments and the isotopic composition of the inorganic and total N determined.

9.3 Results

9.3.1 Composition and nitrogen yields of the legume-grass associations

Clover made up a larger proportion of the total dry matter yield when associated with phalaris than with ryegrass. These proportions for clover were higher than those for lucerne irrespective of the associated grass species (Table 9.1). Similarly, N assimilation by clover was considerably higher ($P < 0.01$) than that for lucerne (Table 9.1). The associated grass had no significant effect on N assimilation by either legume. The addition of ^{15}N-labelled KNO_3 at 1 kg N ha^{-1} had no significant effect on the N accumulated by the legumes or their associated grasses (Table 5.3). Also there was no significant effect of the level of water application or sampling method on the amount of N in the shoots of clover or ryegrass (Table 9.2). When grown together, the N assimilation rate for clover was greater than that for ryegrass (Fig. 9.1).

Table 9.1. Dry matter (DM) and nitrogen yield of shoots for legume/grass associations during 25 days of regrowth after defoliation. Each value is the mean of twelve replicates (six from quadrat data and six from core data).

Legume	Reference plant	Legume DM (% of total)	Legume N ($g\ m^{-2}$)	Grass N ($g\ m^{-2}$)
Lucerne	Ryegrass	24.1	1.00	1.59
	Phalaris	30.9	0.80	0.96
Clover	Ryegrass	52.6	2.41	1.17
	Phalaris	73.1	2.61	0.78
	S.E.D.	4.1	0.38	0.26

Table 9.2. Effect of sampling method, amount of added water and plant part on the yield of N (g m^{-2}) in subterranean clover or annual ryegrass. Each value is the mean of six replicates.

	Sampling method	Water added (mm)	Plant part	Days after ^{15}N addition 10	25	36
Clover:						
	Quadrat	10	Shoots	2.61	4.51	5.53
	Quadrat	2	Shoots	2.82	4.26	5.43
	Core	2	Shoots	3.08	3.89	5.00
	Core	2	Roots	0.83	1.04	1.27
			S.E.D.	0.32	0.30	0.37
Ryegrass:						
	Quadrat	10	Shoots	2.64	2.96	3.43
	Quadrat	2	Shoots	2.31	2.92	3.61
	Core	2	Shoots	2.28	2.72	3.39
	Core	2	Roots	0.78	0.85	0.79
			S.E.D.	0.21	0.24	0.30

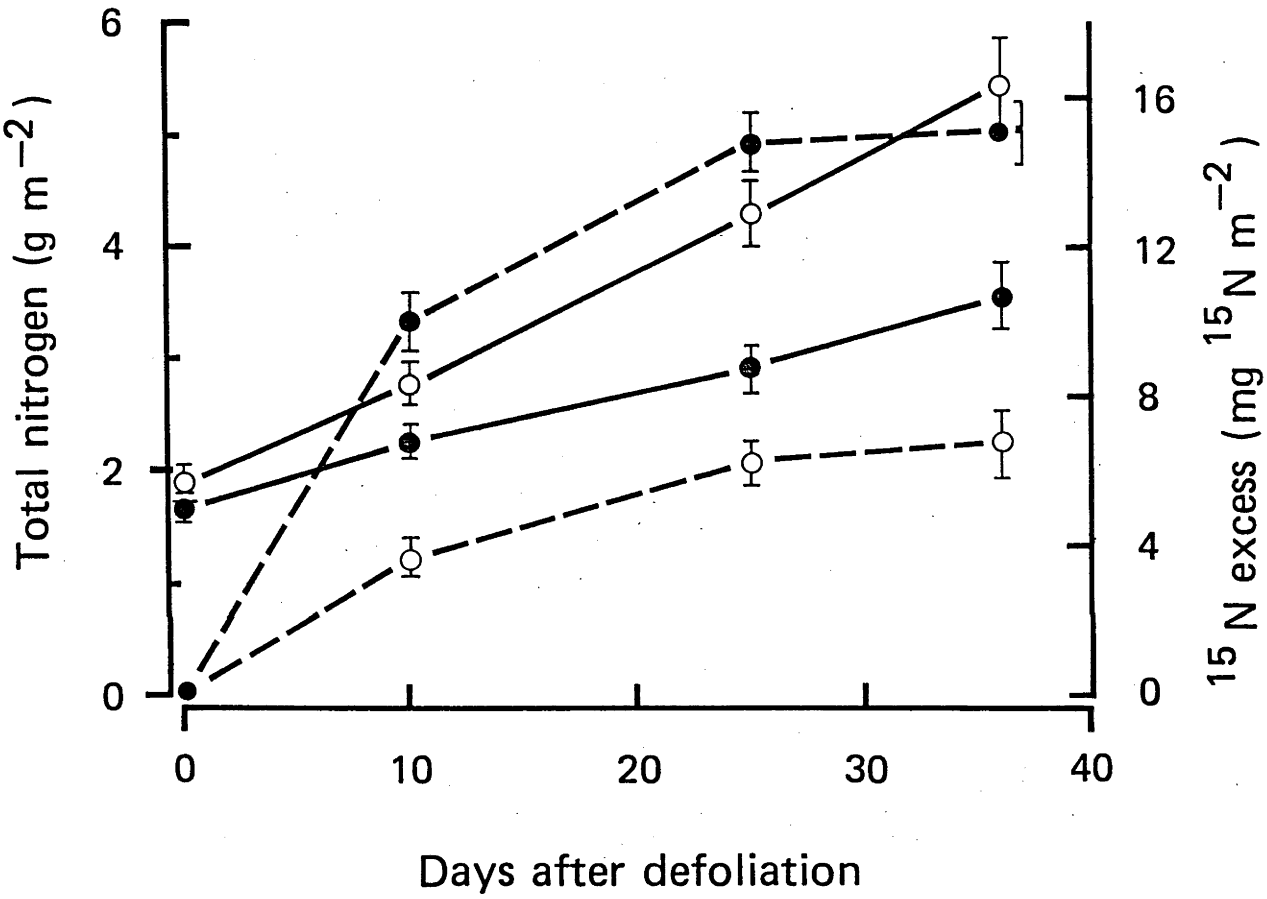


Figure 9.1. Total nitrogen (——) and ^{15}N excess [– – –, calculated as g N m^{-2} x (atoms % $^{15}N_{sample}$ - 0.3663) x 10] in subterranean clover (○) and annual ryegrass (●) shoots. Bars indicate ± 1 S.E.

9.3.2 Effects of sampling procedure on estimation of P

The natural abundance of ^{15}N in clover roots was significantly higher than that in the shoots at all harvests, but there was no significant difference between shoots and roots of ryegrass at any harvest (Table 9.3). In the ^{15}N enriched subplots, the ^{15}N concentration in the shoots of ryegrass was significantly higher than that in the roots when summed over all harvests, but there was no significant difference between the shoots and roots of clover or ryegrass at any one harvest (Table 9.3). In spite of these differences, there was no significant difference in the P values determined with data for whole plants or shoots alone, using natural ^{15}N abundance (Table 9.4a) or ^{15}N isotope dilution (Table 9.4b).

The estimates of plant N yield and P were not affected by the sampling method (core vs. quadrat). However, the errors associated with plant N yields from the quadrat data were lower than those from the core data (by about 40%). A similar trend occurred with the errors associated with P values when either natural abundance (Table 9.4a) or ^{15}N isotope dilution (Table 9.4b) data were used.

9.3.3 Effect of calculation method on the estimate of P

Although yield-dependent estimates of P (equation 34, Chapter 3) were always higher than estimates using the conventional method of calculation (equation 32, Chapter 3), the differences were not significant (Table 9.5). Errors associated with P were higher when yield-dependent calculations were used due to the inclusion of data on N yields and their associated errors. A very large error was associated with the yield-dependent estimate of P during the 25-36 day period, when there was only a very small increase in ^{15}N uptake by plants (Table 9.6).

Table 9.3. Isotopic composition of total nitrogen in roots and shoots of subterranean clover and annual ryegrass from control (natural abundance) and ^{15}N enriched plots. Values for each harvest date are means of six replicates.

	Natural abundance $\delta^{15}N$ (‰)[1]		^{15}N enriched atoms % ^{15}N	
	Clover	Ryegrass	Clover	Ryegrass
Day 10:				
Shoots	2.00	4.71	0.5077	0.8133
Roots	3.49	5.42	0.4462	0.5978
S.E.D.	0.32	0.36	0.0318	0.1359
Day 25:				
Shoots	1.81	4.57	0.5042	0.8529
Roots	2.89	5.13	0.5042	0.7206
S.E.D.	0.40	0.42	0.0379	0.0787
Day 36:				
Shoots	1.50	4.48	0.4850	0.7823
Roots	2.98	4.43	0.4448	0.5881
S.E.D.	0.42	0.38	0.0200	0.1134
Mean:				
Shoots	1.77	4.59	0.4990	0.8162
Roots	3.12	4.99	0.4651	0.6355
S.E.D.	0.22	0.24	0.0196	0.0731

[1] With respect to atmospheric N_2.

Table 9.4. Yield-dependent estimates of the proportion ($\underline{P}$) of clover nitrogen fixed (equation 34, Chapter 3) using a) natural ^{15}N abundance or b) ^{15}N isotope dilution as influenced by method of harvest. The reference plant was annual ryegrass. Each value is the mean of six replicates and values in parentheses are standard errors. Subscripts s and w indicate values calculated from shoots-only and whole plants data, respectively.

Measurement period (days)	Quadrat $\underline{P}_s$ (%)	Core $\underline{P}_s$ (%)	Core $\underline{P}_w$ (%)	Core $\underline{P}_w - \underline{P}_s$ (%)
a) Natural ^{15}N abundance:				
0-10	92.2 (11.9)	79.6 (15.7)	70.3 (17.3)	-9.3 (23.3)
0-25	103.5 (11.3)	85.7 (12.0)	76.4 (14.0)	-9.3 (18.4)
0-36	87.9 (8.4)	87.5 (11.8)	85.5 (12.3)	-2.0 (17.0)
b) ^{15}N isotope dilution:				
0-10	72.3 (10.4)	78.9 (11.8)	74.1 (13.3)	-4.8 (17.6)
0-25	78.8 (6.0)	77.7 (9.6)	78.6 (11.5)	0.9 (14.8)
0-36	80.3 (7.0)	79.3 (7.1)	72.9 (9.1)	-6.4 (11.4)

Table 9.5. Comparison of yield-dependent (Y-D; equation 34, Chapter 3) and conventional (Conv.; equations 31 and 32, Chapter 3) calculations of the percentage of clover nitrogen fixed using natural ^{15}N abundance and ^{15}N isotope dilution methods. The reference plant was annual ryegrass. Data are from quadrats and each value is the mean of six replicates.

Measurement	Natural ^{15}N abundance			^{15}N isotope dilution		
period (days)	Y-D	Conv.	S.E.D.	Y-D	Conv.	S.E.D.
0-10	92.2	72.3	17.3	72.3	64.4	12.1
0-25	103.5	85.2	12.0	78.8	70.4	7.7
0-36	87.9	71.5	10.1	80.3	71.6	8.8

Table 9.6. Yield-dependent estimates of the percentage of clover nitrogen fixed (equation 34, Chapter 3) as influenced by time of application of $K^{15}NO_3$. The reference plant was annual ryegrass and data are from quadrats. Each value is the mean of six replicates and values in parentheses are standard errors.

Measurement period (days)	Time of N application		
	day 0	day 10	day 25
0-10	72.3 (10.4)		
10-25	81.9 (10.4)	85.4 (6.9)	
25-36	84.1 (47.0)		85.2 (11.1)

The estimates of P were similar, whether calculated from the atoms % ^{15}N of clover and ryegrass from the individual plots or by using the mean values for the atoms % ^{15}N over all plots to obtain a mean P value (Table 9.7). Similarly, there was little difference in the errors associated with P between these two methods of calculation (Table 9.7).

9.3.4 Effect of timing and method of ^{15}N application on estimation of P

The isotopic composition of plant N changed with time after ^{15}N application, reaching a maximum on day 25 (Fig. 9.2a). There was no significant increase in ^{15}N uptake after day 25 (Fig. 9.1). This contrasts with the treatment in which ^{15}N was not applied until day 25, when all uptake of ^{15}N occurred during the 25-36 day period. However, despite these differences in time of ^{15}N addition and pattern of ^{15}N uptake during growth there was no significant effect on the estimate of P at day 36 (Table 9.8).

Application at regular intervals of smaller amounts of ^{15}N during the measurement period resulted in no significant difference in the isotopic composition (Table 9.9) of clover or ryegrass at day 36, nor in the estimate of P (Table 9.8), compared with that for one initial larger application.

The application of the ^{15}N with 10 mm of water resulted in a higher estimate of P than with 2 mm (Table 9.8). However, this difference diminished with time. This effect was associated with the ryegrass being more ($P < 0.01$) enriched in ^{15}N at day 10 in the 10 mm than in the 2 mm water treatment. This difference was less at day 25 and had disappeared by day 36. There was no significant

Table 9.7. Effect of method of calculation on the estimates of the proportion ($\underline{P}$) of clover nitrogen fixed using the conventional equations (31 and 32, Chapter 3). Estimates were obtained by calculating $\underline{P}$ for each individual plot or from the mean values for the atoms % ^{15}N of clover and ryegrass nitrogen. Data are from quadrats and each value is the mean of six replicates.

Measurement	Individual plots		Mean data	
period (days)	$\underline{P}$ (%)	S.E.$_{\underline{P}}$	$\underline{P}$ (%)	S.E.$_{\underline{P}}$
Natural abundance:				
0-10	73.1	5.4	72.3	7.4
0-25	85.4	3.2	85.2	4.1
0-36	69.8	6.7	71.5	5.7
^{15}N enriched:				
0-10 (2 mm)[1]	61.1	7.7	64.4	6.2
0-10 (10 mm)[2]	79.9	2.5	80.6	2.5
0-25 (2 mm)	67.6	7.2	70.4	4.9
0-25 (10 mm)	81.6	3.0	82.6	3.0
0-36 (2 mm)	68.4	5.5	71.6	10.8
0-36 (10 mm)	72.9	3.4	75.9	5.2
0-36 (4N)[3]	70.6	4.9	72.9	6.9

[1] 1 kg N ha^{-1} applied on day 0 with 2 mm H_2O.

[2] 1 kg N ha^{-1} applied on day 0 with 10 mm H_2O.

[3] 0.25 kg N ha^{-1} applied on days 0, 9, 18 and 27 with 2 mm H_2O.

Figure 9.2. Atoms % ^{15}N in subterranean clover (○) and annual ryegrass (●) shoots from a) ^{15}N enriched and b) natural ^{15}N abundance plots. Bars indicate ± 1 S.E.

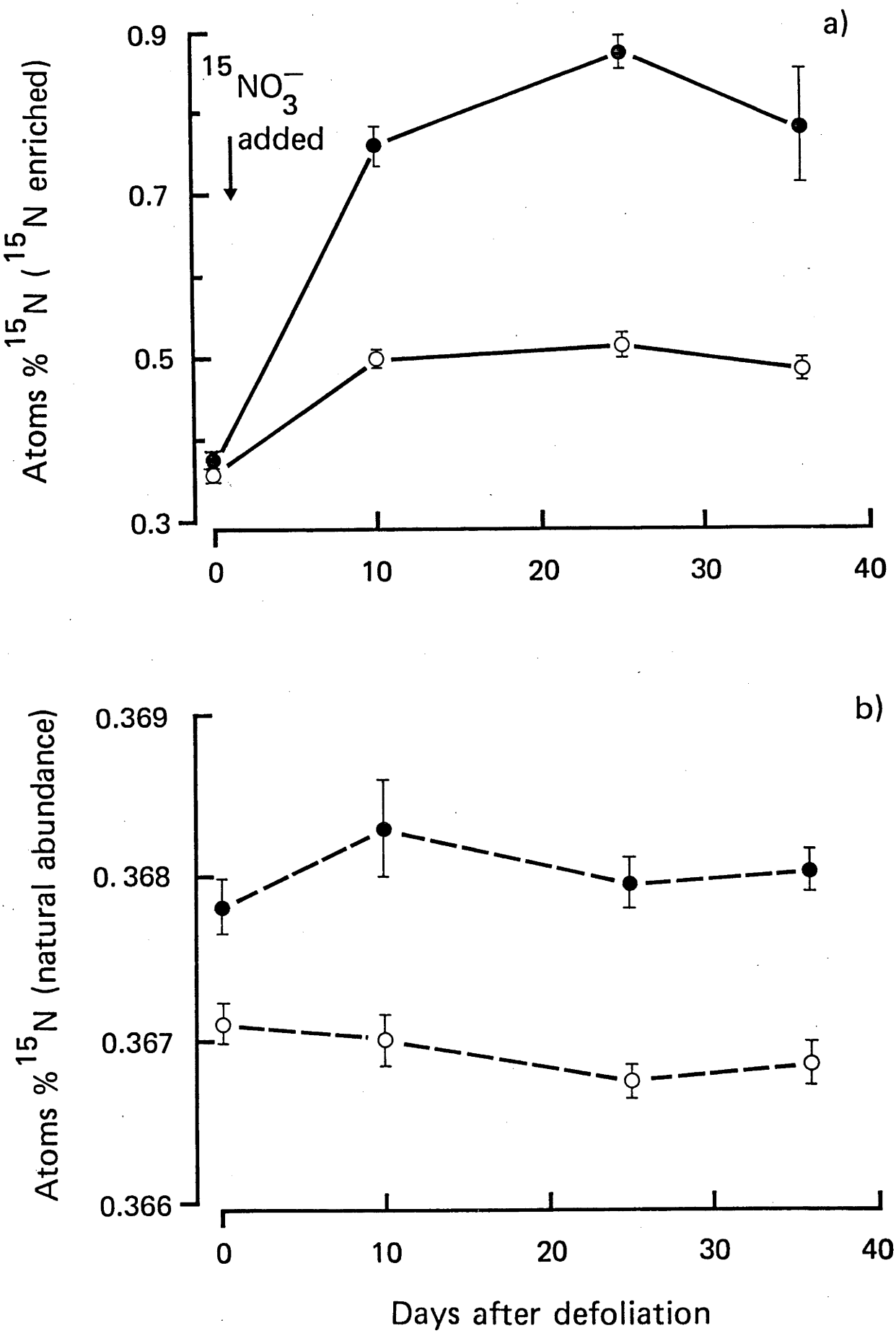

a)
0.9
0.7
0.5
0.3
$^{15}NO_3^-$
added
Atoms % ^{15}N (^{15}N enriched)
0
10
20
30
40
b)
0.369
0.368
0.367
0.366
Atoms % ^{15}N (natural abundance)
0
10
20
30
40
Days after defoliation

Table 9.8. Estimates of the percentage of clover nitrogen fixed using conventional calculations (equations 31 and 32, Chapter 3) as influenced by method of application of $K^{15}NO_3$. The reference plant was ryegrass and data are from quadrats. Each value is the mean of six replicates.

Measurement period (days)	Natural abundance	^{15}N isotope dilution			
		2mm[1]	10mm[2]	4N[3]	S.E.D.[4]
0-10	72.3	64.4	80.6		3.8
0-25	85.2	70.4	82.6		3.3
0-36	71.5	71.6	75.9	72.9	6.4
S.E.D.	7.8	6.2	3.7		

[1] 1 Kg N ha^{-1} applied on day 0 with 2 mm H_2O.

[2] 1 kg N ha^{-1} applied on day 0 with 10 mm H_2O.

[3] 0.25 kg N ha^{-1} applied on days 0, 9, 18 and 27 with 2 mm H_2O.

[4] For comparison between ^{15}N isotope dilution treatments only.

Table 9.9. Effect of method of application of $K^{15}NO_3$ on the atoms % ^{15}N of nitrogen in subterranean clover or annual ryegrass. Data are from quadrats and each value is the mean of six replicates.

	Days after ^{15}N addition					
	10		25		36	
	Grass	Clover	Grass	Clover	Grass	Clover
2 mm[1]	0.7626	0.5076	0.8735	0.5166	0.7840	0.4852
10 mm[2]	1.1086	0.5107	1.1322	0.5000	0.7644	0.4625
4N[3]	-	-	-	-	0.8788	0.5055
S.E.D.	0.0803	0.0210	0.0917	0.0275	0.0696	0.0221

[1-3]See footnotes, Table 9.7.

difference in ^{15}N concentration of clover between the 2 and 10 mm treatments at all harvests (Table 9.9). Analysis of ^{15}N in inorganic and total soil N revealed that the added ^{15}N remained within the 0-50 mm depth where 2 mm water was applied but penetrated into the 50-100 mm layer after a 10 mm application (Table 9.10). The concentrations of inorganic N decreased with soil depth, being 2.37, 1.81, 0.95, 0.63 and 0.44 μg N g^{-1} oven-dry soil for the 0-50, 50-100, 100-150, 150-200 and 200-300 mm depths respectively.

9.3.5 Effect of reference plant on estimation of P

With the ^{15}N isotope dilution method, estimates of P for both legumes were higher ($P < 0.05$) when they were grown with ryegrass than with phalaris (Table 9.11). This was largely due to a higher ($P < 0.05$) ^{15}N concentration in ryegrass than in phalaris (Table 9.11). Estimates of P with the ^{15}N isotope dilution method were also lower ($P < 0.05$) for clover than for lucerne, irrespective of the associated grass. The ^{15}N concentration of clover was higher ($P < 0.05$) when grown with phalaris than with ryegrass and this resulted in the estimate of P for clover grown with phalaris being much lower than for the other legume/grass associations.

Similar estimates of P for lucerne were obtained with the ^{15}N isotope dilution and natural ^{15}N abundance methods. However, P values for clover were lower with the ^{15}N isotope dilution method than with the natural abundance method, particularly when grown with phalaris (Table 9.11). In the clover/phalaris association, the low P value obtained with the ^{15}N isotope dilution method was not due to differential translocation of ^{15}N between the shoots and roots of either plant because estimates of P were similar whether whole plant (P = 43.5%) or shoots-only (P = 45.1%) data was used.

Table 9.10. Effect of method of application of $K^{15}NO_3$ on the atoms % ^{15}N of the inorganic and total nitrogen in the soil after 36 days. Each value is the mean of four replicates.

Soil depth (mm)	Inorganic N			Total N		
	2mm[1]	10mm[2]	4N[3]	2mm	10mm	4N
0-50	0.4308	0.4823	0.5576	0.3784	0.3840	0.3886
50-100	0.3703	0.3873	0.3711	0.3706	0.3772	0.3701
100-150	0.3682	0.3688	0.3689	0.3708	0.3710	0.3703
150-200	0.3693	0.3681	0.3687	0.3709	0.3711	0.3710
200-300	0.3689	0.3688	0.3685	0.3715	0.3717	3.3713
S.E.D.	0.0103	0.0071	0.0072	0.0018	0.0022	0.0026

[1-3]See footnote, Table 9.7.

Table 9.11. Effect of reference plant and ^{15}N method on the ^{15}N concentration of plants and the estimates of the proportion (P) of legume nitrogen fixed using conventional calculations (equations 31 and 32, Chapter 3). Data are for days 0-25 and each value is the mean of six replicates.

Legume	Reference plant	^{15}N isotope dilution Atoms % ^{15}N Legume	Ref.	P (%)	Natural abundance Atoms % ^{15}N Legume	Ref.	P (%)	S.E.D. (P values)
Lucerne	Ryegrass	0.4215	0.8263	88.1	0.36693	0.36793	81.0	5.9
	Phalaris	0.4388	0.6070	70.0	0.36715	0.36793	63.9	6.6
Clover	Ryegrass	0.5166	0.8735	70.4	0.36673	0.36794	85.2	4.8
	Phalaris	0.5615	0.7556	49.9	0.36671	0.36786	85.7	7.4
	S.E.D.	0.0224	0.0554	6.3	0.00010	0.00015	7.4	

There was no significant difference in the natural abundance of ^{15}N in grass N for all legume/grass associations (Table 9.11). Although the errors in estimating the ^{15}N concentration of plant material at natural ^{15}N abundance were low, the differences in ^{15}N concentration between grass and legume were small and therefore the errors in estimating P were higher with the natural abundance method than the ^{15}N isotope dilution method.

Different estimates of P for lucerne were obtained when the weeds, sorrel and chickweed, were used as reference plants instead of ryegrass or phalaris which were growing in the same plot (Table 9.12). This was due to the ^{15}N concentration in the weeds greatly exceeding that of the grasses. The errors associated with these ^{15}N concentrations were similar for the different reference plants, whereas the errors associated with P increased markedly as the P values decreased (Table 9.12).

9.4 Discussion

9.4.1 Effect of sampling procedure on estimation of P

It is considerably easier to obtain samples of plant shoots than of shoots + roots due to the difficulty in obtaining representative root samples free from soil. Thus, it would be simpler to estimate P from data for plant shoots than for whole plants. However, the estimate of P using shoot data may be different from that using whole plant data if the ^{15}N concentrations of the shoots and whole plants are different. In a hypothetical example (Fig. 9.3), it is shown that the greatest error in estimating P from shoots-alone occurred when 1) the ^{15}N concentration of the legume is underestimated by analysing the shoots only and the ^{15}N concentration of the reference plant is overestimated by using data for shoots alone, or when 2) the ^{15}N

Table 9.12. Effect of reference plant on estimation, by ^{15}N isotope dilution (equation 32, Chapter 3), of the proportion ($\underline{P}$) of nitrogen fixed by lucerne, subterranean clover and suckling clover grown together. Each atoms % ^{15}N value is the mean of two replicates.

Major species sown in plot	Reference plant	Atoms % ^{15}N in reference plant	$\underline{P}$[1](%)	S.E. of $\underline{P}$
Lucerne, ryegrass	Ryegrass	0.7282	82.8	4.6
	Sorrel	2.1706	96.8	0.6
	Chickweed	6.5144	98.5	0.4
Lucerne, phalaris	Phalaris	0.6309	74.5	5.6
	Sorrel	1.6651	94.1	0.7

[1]Values are the means for the three legumes (which were not significantly different within each comparison).

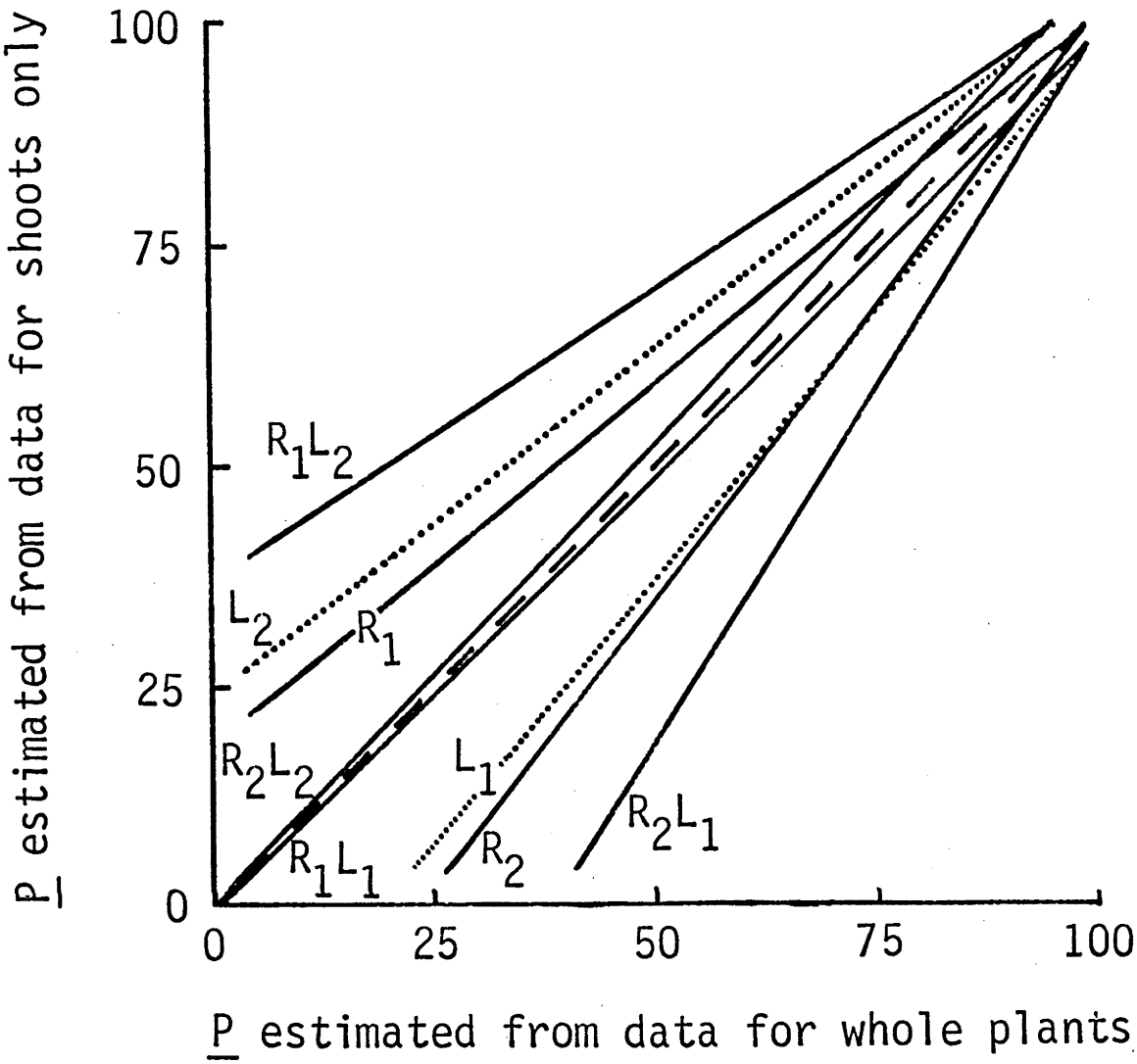


Figure 9.3. Effect of analysis of shoots or whole plants on the estimation of the proportion ($\underline{P}$) of legume nitrogen fixed with various combinations for legume (L) and reference plant (R). In this example, a value for the ^{15}N concentration of the whole reference plant of 5‰ was used. Subscript number 1 indicates a $\delta^{15}N$ of the shoots of 20% higher than that of the whole plants whereas 2 is 20% lower.

concentration of the legume is overestimated and that for the reference plant is underestimated by using data for shoots only. Where there is a similar difference in ^{15}N concentration between the shoots and whole plants for both the legume and reference plant, use of data for shoots alone will have little effect on the estimate of P. The latter result was observed in the field experiment where the natural abundance of ^{15}N in the shoots of both the legume and reference plant was generally less than that for the whole plants and there was little effect on the estimate of P. In pot experiment 3 (Chapter 10), the ^{15}N concentration in the shoots of ryegrass was greater than that in the whole plants at natural abundance and following ^{15}N enrichment, whereas there was no difference between the shoots and whole clover plants. In that case also, there was no effect on the estimate of P.

The effect of differences in ^{15}N concentration between shoots and whole plants on the estimate of P decreases as P increases (Fig. 9.3). Thus, at the high P values obtained in the field experiment and in pot experiment 3, even quite large differences in ^{15}N concentration between the shoots and whole plants would have had little effect on the estimate of P. The field and pot measurements illustrate that a sufficiently accurate estimate of P may be obtained from analysis of the shoots-alone. However, the roots contain about 20% of the total legume N (e.g. Table 9.2) and this should be measured if an accurate estimate of the total amount of N_2 fixed is to be obtained.

9.4.2 Effect of calculation method on the estimate of P

In established legume/grass pastures there is always some plant N present at the start of a period of measurement of N_2 fixation and it has been suggested that this plant N should be excluded from the estimate of P by using yield-dependent calculations (Haystead and Lowe 1977; Bergersen and Turner 1983).

Examples using hypothetical data and data from the 0-10 day measurement period for the clover/ryegrass association, are used to illustrate the factors which result in the initial plant N causing incorrect estimates of P by the conventional method of calculation (equation 32, Chapter 3). Figure 9.4 shows that incorrect estimates of P, using equation 32, are obtained only when the ^{15}N concentration of N assimilated from the soil during the measurement period is at or near natural abundance. This effect decreases 1) as the amount of N assimilated by the legume and reference plant increases and 2) as the difference between the initial P, and that pertaining to the measurement period, decreases. However, in this example (Fig. 9.4) there is no difference between the legume and reference plant in the initial N and in the amount of N assimilated during the measurement period. A major factor affecting the accuracy of the conventional estimate of P (Fig. 9.5) is the ratio (r) of the amounts of N assimilated by the legume (L) and reference plant (R) during the measurement period (N_1-N_o) relative to the initial plant N (N_o), i.e.

$$r = \frac{{}^{L}N_1 - N_o}{{}^{L}N_o} \Big/ \frac{{}^{R}N_1 - N_o}{{}^{R}N_o} \qquad (43)$$

As r deviated from 1, the difference between the conventional (equation 32, Chapter 3) and yield-dependent (equation 34, Chapter

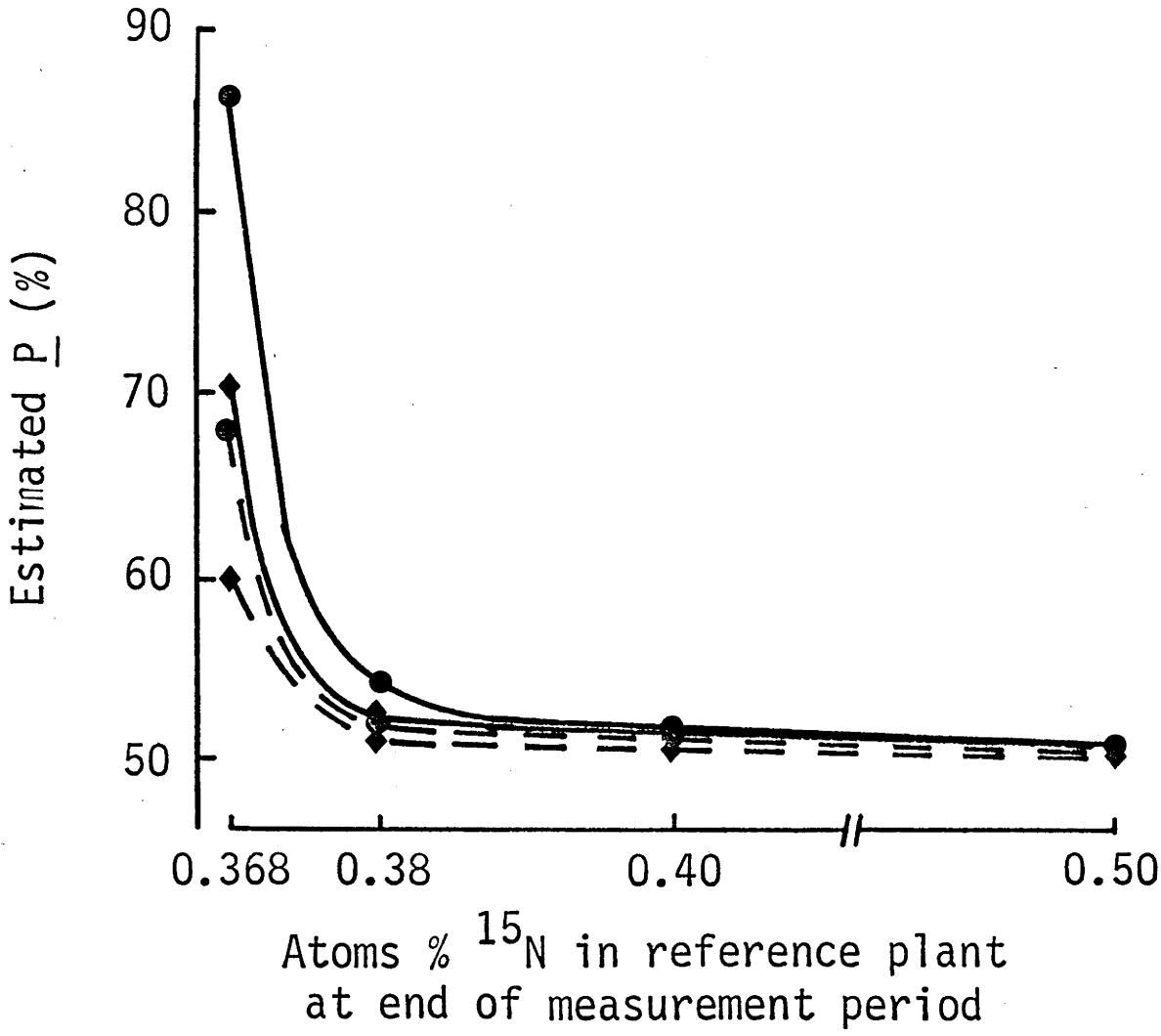
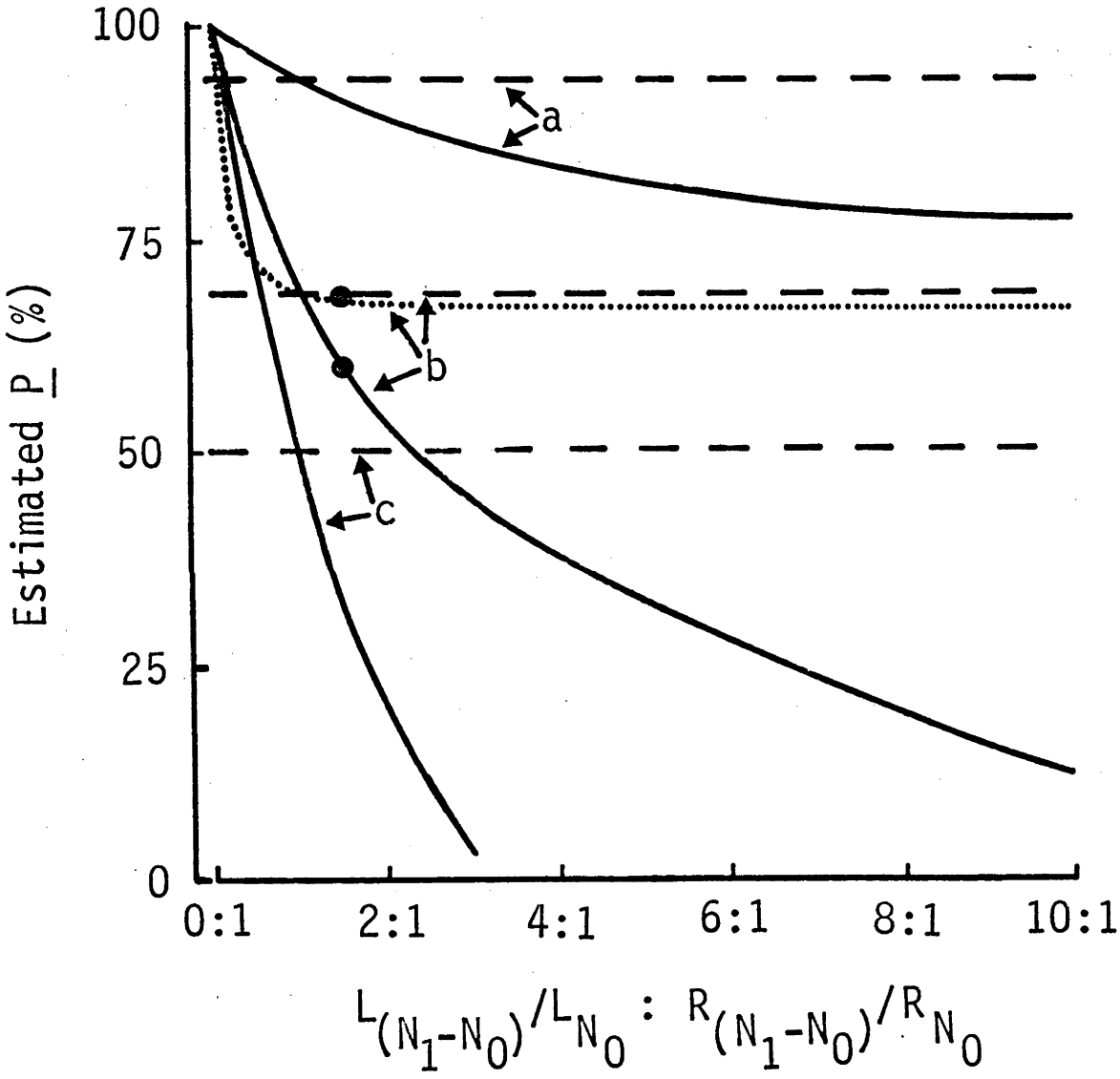


Figure 9.4. Effect of plant nitrogen present at the start of a period of assessment of the proportion (P) of legume nitrogen fixed as influenced by the ^{15}N concentration of nitrogen in the reference plant at the end of that period. It was assumed that the nitrogen assimilated by the legume and reference plant were equal and that the initial P value was 90% (——) or 70% (– –), while P during the measurement period was 50%. Estimates are based on a 10% (●) or 100% (◆) increase in plant nitrogen during the measurement period.

Figure 9.5. Effect of differences in the nitrogen accumulated during the measurement period (N_1-N_0) relative to the initial nitrogen (N_0), between the legume (L) and reference plant (R), on the estimate of the proportion (P) of legume nitrogen fixed. Yield-dependent (– –; equation 34, Chapter 3) and conventional (—— or ····· ; equation 32, Chapter 3) calculations were used on plant nitrogen data from the 0-10 day period of the field experiment, except in one example (·····) where an increase in NR_1 of 13.8 x NR_0 was used (in all other examples, NR_1 = 1.38 x NR_0). Three P values of a) 92, b) 72 and c) 50% were used and the actual values measured in the field experiment using ^{15}N isotope dilution are encircled. A value for the atoms % ^{15}N of R at N_1 of 0.7626 (corresponding with that of the field experiment) was used in all calculations.


100
75
50
25
0
Estimated P (%)
a
b
c
0:1
2:1
4:1
6:1
8:1
10:1
L(N1-N0)/LN0 : R(N1-N0)/RN0

3) estimates of P increased, particularly when the actual value of P approached 50% (Fig. 9.5). However, this effect is negligible when the amount of N assimilated during the measurement period is large relative to the initial plant N (Fig. 9.5).

In the field experiment there was a 53% increase in clover N during the 0-10 day period whereas ryegrass N increased by 38% during this period (Fig. 9.1.) This r of 1.4 resulted in an overestimation of the P value by 8% when the conventional ^{15}N isotope dilution calculation was used (see Table 9.5 and Fig. 9.5). When natural ^{15}N abundance data were used for the conventional calculation of P (equation 31, Chapter 3), the overestimation was 20% (Table 9.5). However, these differences were within field experimental error. Bergersen and Turner (1983) also found no significant differences between yield-dependent and conventional estimates of P for subterranean clover growing with perennial ryegrass, despite relatively large differences in their N assimilation rates. However, in their experiments, P was generally > 90% and it can be seen from Fig. 9.5 that at this level of fixation the effect of r on the accuracy in estimating P by conventional methods is relatively small.

These results and model calculations suggest that it is preferable to use yield-dependent calculations to estimate P where short-term measurements are made, i.e. where the initial plant N is a significant proportion of the plant N at the end of the measurement period. This applies particularly where P < 90%. However, for long-term measurements, the conventional calculations (equations 31 and 32, Chapter 3) can provide an accurate estimate of P.

There was little difference in the error associated with P when estimated from individual plot data or from data for the mean atoms %

^{15}N values. This contrasts with the results of Bergersen and Turner (1983) who found that errors were lower when P was based on individual plot estimates. They attributed their results to variability in the isotopic composition of plant-available soil N between plots; this being largely overcome by estimating P for each individual plot where grass and clover roots assimilate the same N source. Before deciding which calculation method to use for estimating P for a particular site, it may be worthwhile making such a comparison.

9.4.3 Effect of timing and method of ^{15}N application on estimation of P

Differences in the timing and frequency of ^{15}N application imposed on the ryegrass/clover association resulted in marked differences with time in the isotopic composition of the plant-available soil N. However, these treatments had no effect on the estimate of P. This indicates that the pattern of assimilation of soil N with time was similar for clover and ryegrass.

Application of 1 kg N ha^{-1} as KNO_3 (66 atoms % ^{15}N) provided a sufficiently high ^{15}N enrichment in the soil for about 25 days; for longer periods of measurement it would be necessary to use a greater ^{15}N enrichment of the added N, a greater rate of N application or to make successive applications. In Chapter 7 it was shown that higher rates of N application can depress N_2 fixation by the legume and therefore it may be preferable to use successive small additions of ^{15}N-labelled N. This would also serve to give a more uniform ^{15}N concentration in the plant-available soil N with time.

Witty (1983b) used model calculations to illustrate that even relatively small differences in the patterns of N assimilation by the

legume and reference plant could have a large effect on the estimate of P when the ^{15}N concentration in the plant-available soil N varied with time. To overcome this problem, Witty and Ritz (1984) used ^{15}N compounds with slow-release characteristics to give a more uniform ^{15}N concentration in the soil throughout the measurement period. In established pastures, the use of these slow-release compounds might not be worthwhile without incorporation into the soil. If they were placed on the soil surface there may be little penetration of ^{15}N into the soil and only the surface roots would be involved in uptake. Ideally, the soil layers that are explored by plant roots should be uniformly labelled (Knowles 1981).

When $K^{15}NO_3$ was added to the soil in a large volume of water the $^{15}NO_3^-$ was washed further down the soil profile than when a small volume of water was used. The increased volume of water induced a greater ^{15}N uptake by ryegrass and this increased the estimate of P. However, it is not possible to say which of the two estimates of P is correct.

9.4.4 Effect of reference plant on estimation of P

Using the ^{15}N isotope dilution method, the estimates of P for lucerne and clover were lower when they were grown with phalaris than with ryegrass.

These estimates for clover were different from those obtained with the natural ^{15}N abundance method and may be incorrect. In particular, the P value (49.9%) for clover grown with phalaris using the ^{15}N enrichment technique appears to be anomalously low compared with estimates of P for clover species grown with grass by others (Haystead and Lowe 1977; Edmeades and Goh 1978; Goh et al. 1978;

Phillips and Bennett 1978; Bergersen and Turner 1983). However, it is feasible that ryegrass induced the associated legume to fix a larger proportion of its N from atmospheric N_2 than phalaris, due to its greater N assimilation rate and therefore its greater competition for plant-available soil N. To check the accuracy of the P value using ^{15}N isotope dilution, it is necessary to calculate and compare the relative uptake of added N and indigenous soil N by the legume and grass.

The different estimates of P for lucerne using the ^{15}N isotope dilution method with different reference plants growing together in the same plot (Table 9.12) illustrate that the reference plant can cause erroneous estimates of P. This may be caused by differences between reference plants in their level of N uptake with soil depth, in association with differences with depth in the isotopic composition of ^{15}N-labelled plant-available soil N (Edmeades and Goh 1979). Sorrel is known to have a vigorous root system and extensive rhizome development (Whittet 1958) whereas chickweed has a very thin delicate root system (Lamp and Collet 1979). Also, phalaris roots penetrate deep into the soil (McWilliam and Kramer 1968) whereas annual ryegrass has a relatively shallow root system (Rossiter 1966).

Differences in the pattern of N assimilation by legumes and reference plants with time, interacting with changes in the isotopic composition of the plant-available soil N after ^{15}N application, could also cause different estimates of P when different reference plants are used to sample the available soil N pool (Witty 1983b). The competitive success of chickweed is considered to be partly due to its rapid growth rate (Holm et al. 1977) and this species had the highest ^{15}N concentration of all of the reference plants.

These results indicate that the major potential source of error in estimating P by the ^{15}N isotope dilution method is the uncertainty associated with measurement of the ^{15}N concentration of the N assimilated from the soil by the legume using a reference plant. Thus, with this method it is important to select a reference plant to match the legume in its root distribution with soil depth and in its growth pattern with time.

With the natural ^{15}N abundance method, variability in the isotopic composition of soil N with depth and with time are unlikely to have produced errors in the estimate of P. In Chapter 4, it was shown that the isotopic composition of plant-available soil N was uniform throughout the 0-600 mm depth of this soil. The natural abundance of ^{15}N in the soil N assimilated by ryegrass was also found to be constant throughout the measurement period and there was no difference in the natural ^{15}N abundance of ryegrass and phalaris in all legume/grass associations. This uniformity in the isotopic composition of plant-available soil N with soil depth and with time in this experiment support the validity of the natural ^{15}N abundance method for estimating N_2 fixation by legumes in the field.

CHAPTER 10

RELATIVE UPTAKE OF ADDED AND INDIGENOUS SOIL NITROGEN BY LEGUMES AND REFERENCE PLANTS

10.1 Introduction

A major requirement of the ^{15}N isotope dilution technique is that the legume and reference plant assimilate added ^{15}N-labelled N and indigenous soil N in the same ratio (R) (McAuliffe et al. 1958). This requirement may be in doubt due to changes in the isotopic composition of the ^{15}N-labelled plant-available soil N with time (Witty 1983b) and with soil depth (Knowles 1981).

Witty (1983b) suggested using more than one reference plant to obtain an estimate of the isotopic composition of the N assimilated from the soil by the legume. However, in the field experiment (Chapter 9), different estimates of P were obtained when ryegrass or phalaris were grown in association with subterranean clover, and it is not possible to say from these results alone which of the estimates was correct, or whether the grass component influenced P. Therefore it is important to be able to assess R for both the legume and reference plant so that the suitability of different reference plants can be examined objectively.

A direct method for measuring R for the legume and reference plant was developed and tested in two pot experiments and a soil profile experiment. In the soil profile experiment, subterranean clover was grown with either ryegrass or phalaris in an attempt to explain the reasons for the large differences in P obtained using ^{15}N isotope dilution for these two plant associations in the field experiment.

10.2 Principles of the method

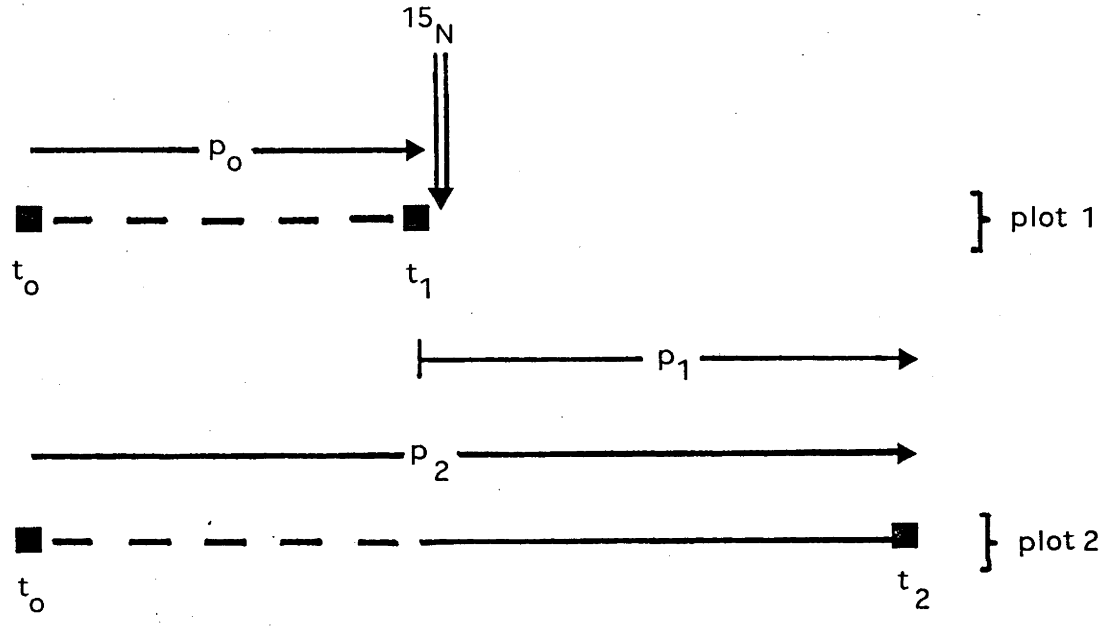
Accurate measurement of the isotopic composition of the three sources of N assimilated by the legume would enable the direct estimation of the amounts and proportions of N derived from each of these sources. In Chapter 4 it was shown that the ^{15}N concentrations of the atmospheric N_2 and soil N could be accurately measured and were sufficiently different to enable the estimation of the amount of N in the legume derived from these two sources. When there is a third N source, added combined N, it is also possible to determine the N derived from all three sources if several treatments are used where the isotopic composition of the added N is varied. This involves a regression approach.

In collaboration with Dr R. Morton of the CSIRO Division of Mathematics and Statistics, Canberra, the following equations were developed to assess the amounts of N derived from the atmosphere, soil and added N by the legume and reference plant. From these results, separate estimates were made of the proportions of added N and indigenous soil N assimilated by the legume and reference plant.

At least three treatments are required. One of these is a control and the others are obtained from applications of combined N at one rate and with two (or more) concentrations of ^{15}N.

When the legume and reference plant are grown together (e.g. in mixed legume/grass pastures) the data for the calculations are obtained by analysing the legume and reference plant for total N and ^{15}N immediately before N addition (the growth period p_o) being the time interval t_0-t_1, see diagram below). On plot is harvested at this stage and ^{15}N-labelled N is added to another identical plot. At the end of a subsequent period of growth (p_1), the second plot is also harvested.

The time, t_0, may correspond with sowing, or in the case of established pastures, it may be a previous grazing or harvest. The periods of growth and the times of ^{15}N addition and measurement used for calculating R are shown in the following diagram:



Thus, the expressions used for the measurement period following N application (period p_1) are not obtained directly but are calculated as the difference between values for p_o and p_2. If ^{15}N-labelled N is applied prior to, or at the time of sowing the plant, p_o is zero and the calculations are simplified (see below).

When the legume and reference plant are grown in separate plots (as is done with most studies on crop legumes) it is necessary to avoid possible differences in the levels of plant-available soil N between plots. These differences, which might gradually develop, would alter the ^{15}N enrichment of the available soil N if ^{15}N-labelled N is added later. Therefore, ^{15}N-labelled N must be applied at or prior to the time of sowing the legume and reference plant, and the simplified equations are also used.

The following equations for the nodulated legume describe the accumulated plant N at the various stages of the experiment using the symbols listed in Table 10.1. The first subscript signifies the source of the ^{15}N (n for natural ^{15}N abundance or control, e for the ^{15}N enriched added N) and the second subscript signifies the measurement period (see Table 10.1).

a) The amount of legume N and its isotopic composition for p_o are

$$\underline{y}_{no} = a_{no} + \underline{s}_{no} \tag{44}$$

and

$$Y_{no} = (a_{no}A + \underline{s}_{no}S_o)/(a_{no} + \underline{s}_{no}). \tag{45}$$

b) For p_1 the corresponding equations for ^{15}N enriched treatments are

$$\underline{y}_{e1} = a_{n1} + \underline{s}_{n1} + \underline{x}_{e1} \tag{46}$$

and

$$Y_{e1} = (a_{n1}A + \underline{s}_{n1}S_1 + \underline{x}_{e1}X_e)/(a_{n1} + \underline{s}_{n1} + \underline{x}_{e1}). \tag{47}$$

c) For p_2 the equations for the ^{15}N enriched treatments are

$$\underline{y}_{e2} = \underline{y}_{no} + \underline{y}_{e1} \tag{48}$$

and

$$Y_{e2} = (\underline{y}_{no}Y_{no} + \underline{y}_{e1}Y_{e1})/\underline{y}_{e2}. \tag{49}$$

In all cases, equations similar to those above can be written for the reference plant if 'a' is made equal to zero and primes are inserted on all symbols, as indicated in Table 10.1.

The isotopic composition of the legume N at the end of the total growth period (Ye_2) should be linearly related to that of the added N (X_e) (e.g. see Fig. 10.1) i.e.

$$Ye_2 = \alpha + \beta X_e \quad , \tag{50}$$

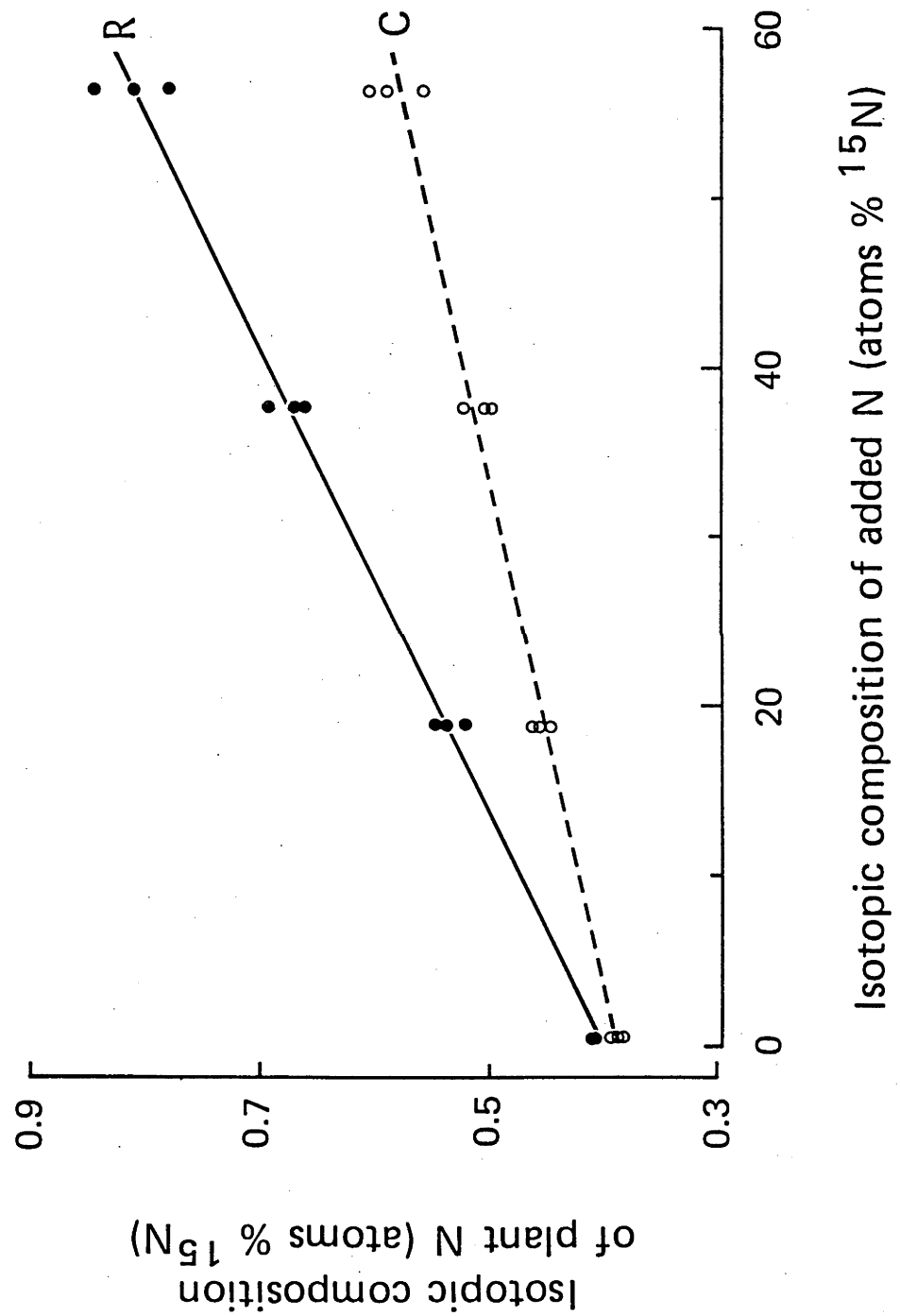
where α and β are the intercept and slope respectively of the regression line. By substituting the terms for $\underline{y}_{no}$, Y_{no}, $\underline{y}_{e1}$ and Y_{e1}

Table 10.1. List of the symbols used to denote the various sources of nitrogen, and its isotopic composition, used by legumes and reference plants during the growth periods before and after addition of ^{15}N-labelled nitrogen to the soil.

Plant species	Nitrogen source	Growth period before N addition (p_o)		Growth period after N addition (p_1)				Total growth period ($p_2=p_o+p_1$)			
		Natural[1]		Natural		Enriched[2]		Natural		Enriched	
		Plant nitrogen									
		Yield[3]	Atoms%	Yield	Atoms%	Yield	Atoms %	Yield	Atoms %	Yield	Atoms %
Legume	Atmosphere	a_{no}	A			a_{n1}	A				
	Soil	$\underline{s}_{no}$	S_o			$\underline{s}_{n1}$	S_1				
	Fertilizer					$\underline{x}_{e1}$	X_e				
	Total	$\underline{y}_{no}$	Y_{no}			$\underline{y}_{e1}$	Y_{e1}			$\underline{y}_{e2}$	Y_{e2}
Reference plant	Soil	$\underline{s}'_{no}$	S'_o	$\underline{s}'_{n1}$	S'_1	$\underline{s}'_{n1}$	S'_1				
	Fertilizer					$\underline{x}'_{e1}$	X'^{e}_{1}				
	Total	$\underline{y}'_{no}$	Y'_{no}	$\underline{y}'_{n1}$	Y'_{n1}	$\underline{y}'_{e1}$	Y'^{e}_{e1}	$\underline{y}'_{n2}$	Y'_{n2}	$\underline{y}'_{e2}$	Y'_{e2}

1 Natural ^{15}N abundance i.e. no added N.
2 Addition of a nitrogen compound enriched in ^{15}N at a level e; there being two or more level of enrichment.
3 Nitrogen yield parameter e.g. mgN plant^{-1}, gN m^{-2} etc.

Figure 10.1. Relationship between the isotopic composition of nitrogen in ammonium sulphate (surface-applied at 1 kg N ha^{-1}) and nitrogen in annual ryegrass (R) or subterranean clover (C). The two species were grown together in pots (pot experiment 4). Correlation coefficients for the regression lines were 0.994 for G and 0.982 for C.


R
C
0.9
0.7
0.5
0.3
0
20
40
60
Isotopic composition of added N (atoms % ^{15}N)
Isotopic composition
of plant N (atoms % ^{15}N)

from equations 44, 45, 46, and 47 into equation 49 and rearranging it can be seen that the intercept (α) and slope (β) can be written as follows:

$$\alpha = [(a_{no} + a_{n1})A + \underline{s}_{no}S_o + \underline{s}_{n1}S_1]/\underline{y}_{e2} \quad (51)$$

and

$$\beta = \underline{x}_{e1}/\underline{y}_{e2} \quad . \quad (52)$$

The corresponding relationship for the reference plant (e.g. Fig. 10.1) is

$$Y'_{e2} = \alpha' + \beta' X'_e \quad , \quad (53)$$

where

$$\alpha' = (\underline{s}'_{no}S'_o + \underline{s}'_{n1}S'_1)/\underline{y}'_{e2} \quad , \quad (54)$$

and

$$\beta' = \underline{x}'_{e1}/\underline{y}'_{e2} \quad . \quad (55)$$

The legume N derived from the soil during the initial growth period can be eliminated from equations 51 and 45 by substituting for $\underline{s}_{no}$ from equation 44 to give

$$\alpha = [(a_{no} + a_{n1})A + (\underline{y}_{no} - a_{no})S_o + \underline{s}_{n1}S_1]/\underline{y}_{e2} \quad (56)$$

and

$$Y_{no} = [a_{no}A + (\underline{y}_{no} - a_{no})S_o]/\underline{y}_{no} \quad . \quad (57)$$

The atoms % ^{15}N of the legume N derived from the soil during the initial growth period can then be eliminated from the expression for α by rearranging equation 57 and substituting for S_o in equation 56.

Equation 56 then becomes

$$\alpha\, \underline{y}_{e2} = \underline{y}_{no}Y_{no} + a_{n1}A + \underline{s}_{n1}S_1 \quad . \quad (58)$$

Another expression relating a_{n1} and $\underline{s}_{n1}$ can be obtained as follows: The total N yield of the legume, derived during the total growth period (p_2), can be described by the equation,

$$\underline{y}_{e2} = \underline{y}_{no} + a_{n1} + \underline{s}_{n1} + \underline{x}_{e1} \quad . \tag{59}$$

The term $\underline{x}_{e1}$ can be eliminated by substituting the terms from equation 52. Equation 59 then becomes

$$\underline{y}_{e2}(1-\beta) = \underline{y}_{no} + a_{n1} + \underline{s}_{n1} \quad . \tag{60}$$

We then have two simultaneous equations (58 and 60) which can be solved for a_{n1} and $\underline{s}_{n1}$. The solution is

$$a_{n1} = [\{\alpha-(1-\beta)S_1\}\underline{y}_{e2} - \underline{y}_{no}\,(Y_{no}-S_1)]/(A-S_1) \tag{61}$$

$$\underline{s}_{n1} = [\{\alpha-(1-\beta)A\}\underline{y}_{e2} - \underline{y}_{no}\,(Y_{no}-A)]/(S_1-A) \quad . \tag{62}$$

The legume N derived from the added N can be obtained by rearranging equation 52 to give

$$\underline{x}_{e1} = \beta\underline{y}_{e2} \quad . \tag{63}$$

The isotopic composition of the N derived from the indigenous soil N should be the same for the legume and reference plant i.e. $S_1 = S'_1$. Therefore the value for S_1 can be obtained from the atoms % ^{15}N value for the reference plant in the control treatment.

$$S_1 = S'_1 = Y'_{n1} = (\underline{y}'_{n2}Y'_{n2} - \underline{y}'_{no}Y'_{no})/(\underline{y}'_{n2} - \underline{y}'_{no}) \quad . \tag{64}$$

Equations for calculating the amount of N derived from the soil N and added N by the reference plant can also be derived in a similar manner. In this case, as no atmospheric N_2 is fixed, a_{n1} in equations 58 and 60 is zero. Thus, equations 62 and 63 become

$$\underline{s}'_{n1} = (\alpha'\underline{y}'_{e2} - \underline{y}'_{no}Y'_{no})/S_1 \tag{65}$$

and

$$\underline{x}'_{e1} = \beta'\underline{y}'_{e2} \quad . \tag{66}$$

The ratio of added N to soil N absorbed by the legume ($\underline{R}$) can be readily calculated from equations 62 and 63 and the ratio for the reference plant ($\underline{R}'$) from equations 65 and 66. Then,

$$\underline{R} = \underline{x}_{e1}/\underline{s}_{n1} = \beta\underline{y}_{e2}(S_1-A)/[\{\alpha-(1-\beta)A\}\underline{y}_{e2} - \underline{y}_{no}(Y_{no}-A)], \tag{67}$$

and

$$\underline{R}' = \underline{x}'_{e1}/\underline{s}'_{n1} = \beta'\underline{y}'_{e2}\, S'_1/(\alpha'\underline{y}'_{e2} - \underline{y}'_{no}Y'_{no}) \quad . \qquad (68)$$

If the ^{15}N-labelled N is applied at the time of sowing the legume and reference plant, $\underline{y}_{no}$ is equal to zero and the determination of these ratios is greatly simplified. In that case, $\underline{R}$ can be rewritten as:

$$\underline{R}_s = \beta(S_1 - A)/\{\alpha - (1-\beta)A\} \quad . \qquad (69)$$

Similarly, for the reference plant, $\underline{y}'_{no}$ could be taken as zero and $\underline{R}'$ can be rewritten as:

$$\underline{R}'_s = \beta' S'_1/\alpha' \quad . \qquad (70)$$

It is apparent from equations 69 and 70 that the ratios used in the simplified method are independent of the N yields of the legume and reference plant.

This simplified approach can also be used where the legume and reference plant are grown together and the shoot growth is removed by clipping immediately prior to N addition, although with some loss of validity.

10.3 Experimental

10.3.1 Pot experiment 3

The 0-100 mm layer of a yellow podzolic soil (soil 5, Table 3.1) was passed through a 1 mm sieve before use. Its total N concentration was 0.147% with 0.3690 atoms % ^{15}N and its pH was 5.7.

The equivalent of 1.85 kg oven-dry soil per polythene-lined sealed pot (140 mm diameter) was mixed with a basal nutrient solution containing (mg pot^{-1}) KH_2PO_4 (500), Na_2SO_4 (400), $Na_2B_4O_7.10H_2O$ (7.7) and $Na_2MoO_4.2H_2O$ (0.4). Distilled water was added to adjust the soil water content to 70% of field capacity.

Pregerminated seeds of subterranean clover (cv. Woogenellup) and annual ryegrass (cv. Wimmera) were planted together in each pot. A suspension of peat inoculant (Nodulaid C: Agricultural Laboratories, Sefton, N.S.W.) was then sprayed onto the soil around each clover seedling to ensure effective nodulation. Once the plants were established they were thinned to six clover and eight ryegrass plants per pot. The pots were kept in a glasshouse with temperatures controlled at a minimum of 15°C (night) and maximum of 30°C (day). Daily applications of distilled water were made to maintain the soil water near 70% of field capacity.

Thirty four days later, all plants were trimmed to 20 mm above the soil surface, and ten pots were then harvested. Roots and stubble were recovered from the soil by sieving and washing. Nitrogen treatments were applied to the remaining pots. Two rates of $NaNO_3$ (0.46 and 1.54 mg N pot^{-1}, being equivalent to 0.3 and 1.0 kg N ha^{-1}) were used at three concentrations of ^{15}N. These were analysed to be 0.3669, 9.9750 and 59.8500 atoms % ^{15}N. The $NaNO_3$ was applied to the soil surface in solution (10 ml pot^{-1}) and washed into the soil with 50 ml distilled water. There were 12 replicates of a control (no added N) and 8 replicates of each ^{15}N enriched $NaNO_3$ treatment.

Twenty one days after N addition, plant material from all pots was harvested by cutting off the shoots 20 mm above the soil surface and retrieval of plant stubble + roots was as described above. Plant material was separated into clover and grass components and analysed for total N and ^{15}N.

10.3.2 Pot experiment 4

The 0-100 mm layer of another yellow podzolic soil (see section 10.3.1) was collected from pasture plots which had received 1.05 kg N ha^{-1} as $NaNO_3$ (95 atoms % ^{15}N) three years previously. The total N content of this layer was 0.281% (almost twice that of pot experiment 3) with 0.3879 atoms % ^{15}N and its pH was 5.7.

Soil preparation, pots, basal fertilizer solution, plant species and planting procedure were the same as in pot experiment 3. The equivalent of 1.57 kg oven-dry soil was used for each pot which contained three subterranean clover and four ryegrass plants. Ryegrass was planted four days after the subterranean clover in an attempt to obtain a more comparable pattern of growth with time for the two species than occurred in pot experiment 3. Clover plants were removed from surplus pots at regular intervals to check on nodule development. By 30 days after planting, the nodules were well developed and all plants were trimmed to 20 mm height. Six replicates were then harvested.

Two forms of combined N [$NaNO_3$ and $(NH_4)_2SO_4$] were applied to the soil surface (as in pot experiment 3) at one rate (1.54 mg N pot^{-1}, being equivalent to 1 kg N ha^{-1}) and four levels of ^{15}N enrichment. These were analysed to be 0.3742, 19.200, 38.400 and 57.600 atoms % ^{15}N for $NaNO_3$ and 0.3689, 18.750, 37.500 and 56.250 atoms % ^{15}N for $(NH_4)_2SO_4$. There were 16 replicates of a control (no added N), 12 replicates of $NaNO_3$ at 4 concentrations of ^{15}N and 6 replicates of $(NH_4)_2SO_4$ at 4 levels of ^{15}N enrichment. Three replicates of the $NaNO_3$ treatments were harvested 3, 6, 12 and 27 days after N addition and three replicates of the $(NH_4)_2SO_4$ treatments were harvested 6 and

27 days after N addition. In this experiment the harvested plants were not separated into shoots and roots, but the whole plants were used for analysis of total N and ^{15}N.

10.3.3 Soil profile experiment

10.3.3.1 Soils

The same soil as described in section 10.3.2 was used in this experiment except that three layers of soil were collected (0-100, 100-200 and 200-400 mm) from areas with and without the previous ^{15}N enrichment.

Two major studies were conducted within this experiment and these involved different soil combinations. In the main study on the ratio ($\underline{R}$) of uptake of added N and indigenous soil N, the 0-100 mm soil layer had been labelled in ^{15}N three years previously, whereas the 100-200 and 200-400 mm layers were unlabelled. In the root activity study, all three layers were unlabelled. The 0-100 mm labelled soil had a total N concentration of 0.226% N with 0.3760 atoms % ^{15}N and its pH was 5.7. The 0-100, 100-200 and 200-400 mm layers of unlabelled soil contained 0.204, 0.064 and 0.027% N and had ^{15}N concentrations of 0.3680, 0.3691 and 0.3691 atoms % ^{15}N respectively. The pH of these layers was 5.7, 5.4 and 6.5 respectively.

10.3.3.2 Experimental design

10.3.3.2.1 Main study (estimation of R)

A randomised block design was used with two replicates of the following treatments: 2 species combinations x [1 pretreatment harvest + (4 levels of ^{15}N enrichment x 4 harvest dates)]. Subterranean clover was sown with either annual ryegrass or phalaris and ten weeks after sowing, the plant shoots were trimmed to 20 mm height. At this stage

two replicates of each of the two plant combinations were removed for a pretreatment harvest. The remaining treatments then received either no added N, or KNO_3 (5.3 mg N profile^{-1}, being equivalent to 1 kg N ha^{-1}) at three concentrations of ^{15}N (1.0444, 22.9255 and 68.0411 atoms % ^{15}N) added to the soil surface with 290 ml distilled water (equivalent to 2 mm precipitation). Harvests were taken 4, 8, 16 and 32 days after trimming.

10.3.3.2.2 Root activity study

Three replicates of two species combinations (as in section 10.3.3.2.1) and four treatments were used. The treatments were no added N and KNO_3 solution (15 µg N ml^{-1} and 99 atoms % ^{15}N) injected into the soil at eight positions around the pot for each of the 50, 150 and 300 mm soil depths. Harvests were taken 4 and 16 days after ^{15}N injection.

10.3.3.3 Experimental procedure

Coarse river-sand (6 kg) was placed in the bottom of each cylinder (260 mm diameter, 450 mm deep; Plate 10.1) to facilitate water drainage. Soil from each layer was shredded, mixed and placed into the cylinders in each of their respective layers and pressed down until a bulk-density similar to that found in the field was obtained. The amounts of soil used were equivalent to 5.7, 7.4 and 15.5 kg oven-dry soil for the 0-100, 100-200 and 200-400 mm layers respectively.

The cylinders were placed in a glasshouse (6°-25°C) and a basal nutrient solution containing (mg cylinder^{-1}) KH_2PO_4 (1000), Na_2SO_4(600), $MgSO_4.7H_2O$ (200), $Na_2B_4O_7.10H_2O$ (26) and $Na_2MoO_4.2H_2O$ (4) was applied to the soil surface in each cylinder and washed in with distilled water (1 l cylinder^{-1}).

Plate 10.1. Cylinders used in the soil profile experiment showing, from left to right, a subterranean clover/phalaris association and a subterranean clover/annual ryegrass association. The cylinders were located in PVC trays so that a bottom-watering system could be used.

Clover seeds were sprayed with a suspension of peat inoculant (Nodulaid C) to ensure effective nodulation and plant seeds were sown 10 mm below the soil surface. During the first nine weeks after sowing, the soils were given regular applications of distilled water until water drained out of the bottom of the cylinders. At nine weeks, the cylinders were relocated at random within the glasshouse and a bottom-watering system was used to simulate field conditions; the cylinders were placed into PVC trays to which distilled water was added regularly (Plate 10.1). Nine weeks after sowing, soil cores were removed from the surplus cylinders to examine root penetration into the soil. At this stage some roots had penetrated to near the bottom of the cylinder and therefore treatments were commenced one week after beginning bottom-watering.

Separate cylinders were used at each harvest, except in the root activity study where the shoots collected at day 16 were the regrowth since the day 4 harvest. Plants were harvested by cutting off the shoots at ground level. The plant material for the two halves of each cylinder were kept separate. In the main study (estimation of $\underline{R}$), root samples were also collected at days 0 and 16 after trimming by removing two 150 mm diameter cores from each soil layer in each cylinder; the roots were washed with tap water to remove adhering soil. All shoot and root samples were separated into clover and grass components and analysed for total N and ^{15}N.

In the main study, soil samples were collected at 4, 8, 16 and 32 days after trimming from the 0-25, 25-50 (at day 4 only a 0-50 mm sample was collected), 50-100, 100-150, 150-200 and 200-400 mm layers of the control and KNO_3 (68 atoms % ^{15}N) treated cylinders and analysed for inorganic N and ^{15}N.

10.3.3.3.1 Method of ^{15}N injection for the root activity study

The method of ^{15}N injection involved forcing a stainless steel tube (8 mm external diameter) with an internal plunger into the soil to the desired depth. The plunger was removed and the tube was lifted up in the soil by 20 mm to avoid subsequent contamination of the tube by ^{15}N. The ^{15}N solution was drawn into a syringe which had a piece of 2 mm diameter plastic tubing (500 mm long) attached. The plastic tubing was inserted into the stainless steel tube until it was 10 mm below the bottom of the stainless steel tube. Two ml of $K^{15}NO_3$ solution was then injected into the soil. The plastic tubing and the stainless steel tube were then removed. A piece of 8 mm diameter wooden dowel was then forced into the soil channel left by the stainless steel tube to prevent movement of water and roots down the channel.

The volume and number of additions of $K^{15}NO_3$ solution to be used was based on a preliminary study on several surplus cylinders. In this study, 1, 2 or 4 ml of bromocresol blue or phenol red dye was injected into the soil and after four days the zone of penetration was determined. The volume of affected soil increased as the volume of dye increased. When 2 ml was added, the volume of soil affected was 3.2 cm^3 for both dyes. To ensure that the injected ^{15}N would be evenly assimilated by all plants, eight positions of injection were used for each soil depth.

10.3.4 Calculation of the linear regressions

The plant N derived from the atmosphere, soil and added N and the relative uptake of added N and soil N ($\underline{R}$) were calculated by the procedure outlined in section 10.3.1. In that procedure the main parameters required are the intercept (α) and slope (β) for the linear

relationship between the isotopic composition of plant N (Y_i) and that of the added N (X_i for the i-th level of ^{15}N enrichment of the added N). Incorporation of a random error term (E_{ij}) gives the following model for the jth replicate:

$$Y_{ij} = \alpha + \beta X_i + E_{ij} \quad (71)$$

In all three experiments it was found that the variance of the error term (E_{ij}) did not remain constant but increased markedly as the ^{15}N concentration of the added N increased. Thus a weighted least-squares regression (Draper and Smith 1966) was used where the weight (W_i) is

$$W_i = 1/\text{var}\ (E_{ij}) \quad . \quad (72)$$

Variances for R values were obtained using a series expansion to the first order (Davies and Goldsmith 1972).

10.4 Results

10.4.1 Pot experiment 3

There was no significant effect of 0.46 or 1.54 mg N pot^{-1} of $NaNO_3$ on the total N accumulated by clover or grass (Table 7.2). At the time of N addition, the total N yields (roots + stubble) were 1.20 and 3.49 mg N pot^{-1} for subterranean clover and ryegrass respectively. The N assimilation rates over the 21 day growth period following addition of $NaNO_3$ were 0.67 and 1.55 mg N pot^{-1} day^{-1} respectively.

In the control (no added N) treatment, the isotopic composition of the grass N (whole plants) at the start of the growth period was 0.36742 ± 0.00009 atoms % ^{15}N. Twenty one days later this had increased to 0.36906 ± 0.00003 atoms % ^{15}N and 0.36828 ± 0.00003 atoms % ^{15}N for shoots and whole plants respectively.

The linear equations relating the isotopic composition of added N and plant N explained 99.8% of the variation in the data (adjusted for degrees of freedom). Table 10.2 shows the total N yield for grass and clover, derived from the atmosphere, soil and added N. With grass, the N derived from $NaNO_3$ applied at a rate of 0.46 mg N pot^{-1} was only about 1% of the total. Where 1.54 mg N pot^{-1} was applied, the grass N derived from added N increased in direct proportion to the increased application rate (i.e. 3.3 times) for both shoots and whole plants (Table 10.2).

There was no significant difference between the R values for clover and grass at either rate of N addition, whether one considers the whole plant or just the data for the shoots (Table 10.3). With the higher N rate, the errors associated with R were greater than those at the lower rate and this resulted in a high S.E.D. for the comparison with ryegrass. This was due to the error for the slope (β) of the regression (atoms $\%^{15}N$ in added N vs. atoms $\%^{15}N$ in clover N) being large (Table 10.4). The error associated with R was considerably greater for clover than for grass (10-36 times), due mainly to the error associated with the small difference between the isotopic composition of atmospheric and soil N. Also, the error in R for whole plants exceeded that for shoots only (3-5 times) due to the greater number of factors involved in the calculation of R for whole plants.

Similar P values were obtained by using a ^{15}N isotope dilution method (which requires that the R values for the legume and reference plant be the same) and by a natural ^{15}N abundance method (which has no requirement concerning R, since no N was added) (Table 10.5). This suggests that the method used for calculating R is reliable. There was no effect on P of either rate of N addition (Table 10.5). It can be

Table 10.2. Apportioning of nitrogen in subterranean clover and ryegrass into atmospheric, soil and added nitrogen components as influenced by rate of $NaNO_3$ application (pot experiment 3). Values were estimated as described in section 10.2. Each value was calculated from 36 observations.

	Rate of N applied (mg pot^{-1})	Nitrogen source	CLOVER mgN pot^{-1}	S.E.	GRASS mgN pot^{-1}	S.E.
Whole plants	0.46	air	13.29	1.13		
		soil	0.877	0.564	32.25	2.00
		$NaNO_3$	0.0114	0.0011	0.3608	0.0089
	1.54	air	13.60	1.08		
		soil	0.528	0.431	31.44	2.00
		$NaNO_3$	0.0573	0.0051	1.178	0.036
Shoots only	0.46	air	12.762	0.462		
		soil	0.991	0.324	26.132	0.266
		$NaNO_3$	0.00737	0.00074	0.2624	0.0071
	1.54	air	12.849	0.463		
		soil	0.869	0.323	25.545	0.260
		$NaNO_3$	0.0416	0.0037	0.860	0.016

Table 10.3. Ratio of added nitrogen to soil nitrogen (R) assimilated by subterranean clover and annual ryegrass as influenced by rate of $NaNO_3$ addition (pot experiment 3). Each value was calculated from 36 observations.

	Rate of N applied (mg pot^{-1})	Grass R	Clover R	Grass R - Clover R	S.E.D.
Whole plants	0.46	0.01119 (0.00069)[1]	0.01307 (0.0077)	-0.00188	0.00771
	1.54	0.0375 (0.00147)	0.0646 (0.0290)	-0.0271	0.0290
Shoots only	0.46	0.01004 (0.00025)	0.00744 (0.0026)	0.00260	0.00258
	1.54	0.0337 (0.00055)	0.0478 (0.0180)	-0.0141	0.0182

[1] Values in brackets are standard errors. For comparisons between estimates in the same column S.E.D. = $\sqrt{S.E._1^2 + S.E._2^2}$; for comparisons between rows differences involve a covariance term therefore S.E.D.s are provided.

Table 10.4. The intercepts (α) and slopes (β) of the regression lines relating the isotopic composition of plant nitrogen to that of added nitrogen for subterranean clover and annual ryegrass as influenced by rate of $NaNO_3$ addition (pot experiment 3) or form and time of nitrogen addition (pot experiment 4). Each value in pot experiment 3 was calculated from 36 observations and in pot experiment 4, from 16 observations.

	Clover		Ryegrass	
	α	$\beta(\times 10^{-3})$	α	$\beta(\times 10^{-3})$
POT EXPERIMENT 3				
Whole plants:				
0.46 mgN pot^{-1}	0.36654 (0.00005)[1]	0.52 (0.05)	0.36558 (0.00010)	5.96 (0.12)
1.54 mgN pot^{-1}	0.36573 (0.00009)	2.61 (0.22)	0.36063 (0.00020)	19.44 (0.54)
Shoots only:				
0.46 mgN pot^{-1}	0.36651 (0.00006)	0.54 (0.05)	0.36421 (0.00019)	9.91 (0.25)
1.54 mgN pot^{-1}	0.36557 (0.00011)	3.02 (0.26)	0.35603 (0.00020)	32.48 (0.52)
POT EXPERIMENT 4				
$NaNO_3$:				
Day 3	0.3956 (0.0010)	2.32 (0.25)	0.3998 (0.0010)	5.28 (0.25)
Day 6	0.3946 (0.0010)	4.63 (0.25)	0.3973 (0.0010)	9.99 (0.33)
Day 12	0.3944 (0.0010)	8.67 (0.25)	0.3964 (0.0010)	11.32 (0.27)
Day 27	0.3871 (0.0010)	5.32 (0.25)	0.4005 (0.0010)	10.13 (0.25)
$(NH_4)_2SO_4$:				
Day 6	0.3943 (0.0010)	3.48 (0.26)	0.3966 (0.0010)	9.08 (0.26)
Day 27	0.3883 (0.0013)	3.33 (0.27)	0.4017 (0.0013)	7.16 (0.27)

[1]Values in brackets are standard errors. To obtain comparisons between estimates within rows, S.E.D. $= \sqrt{S.E._1^2 + S.E._2^2}$.

Table 10.5. Estimates of the proportion (P) of clover nitrogen fixed using data from natural ^{15}N abundance and ^{15}N-enriched treatments. In pot experiment 4, day 27 data were used. The number of observations used to calculate each value in pot experiment 3 was 12 and 24, and in pot experiment 4 was 4 and 12, for natural abundance and +N treatments, respectively.

Pot experiment 3		Pot experiment 4	
Treatment	P(%)	Treatment	P[1](%)
Whole plants[1]			
natural ^{15}N abundance	95.56	natural ^{15}N abundance	42.41
+ 0.46 mg N pot^{-1}	91.60	+ ^{15}N enriched $NaNO_3$	43.03
+ 1.54 mg N pot^{-1}	90.78	+ ^{15}N enriched $(NH_4)_2SO_4$	51.04
S.E.D. (nat.ab.*vs*.+ N)	3.95	S.E.D. (nat.ab.*vs*. + N)	2.90
S.E.D. (between N levels)	2.08	S.E.D. (NO_3^- *vs*. NH_4^+)	2.75
Shoots only[2]:			
natural ^{15}N abundance	94.32		
+ 0.46 mg N pot^{-1}	91.59		
+ 1.54 mg N pot^{-1}	89.72		
S.E.D. (nat.ab.*vs*. + N)	2.69		
S.E.D. (between N levels)	1.41		

[1] P values based on yield-dependent calculations (equation 34, Chapter 3).

[2] P values based on conventional calculations (equations 31 and 32, Chapter 3).

seen from Table 10.6a that there was no significant difference in the estimates of the amount of N_2 fixed by clover using natural ^{15}N abundance, ^{15}N isotope dilution or the method proposed in section 10.2 (equation 61).

10.4.2 Pot experiment 4

As with pot experiment 3, an application of 1.54 mg N pot^{-1} did not affect the N yield of clover or grass. In contrast to pot experiment 3, the N yields for clover were greater than those for the associated grass and except for the period up to day 3 the N assimilation rate for clover exceeded that for grass (compare Figs. 10.2a and 10.2b).

Where no labelled-N was applied, the grass N contained about 0.40 atoms % ^{15}N and this remained relatively constant during the experimental period (Fig. 10.3). The linear equations relating the isotopic composition of added N and plant N explained 98.6% of the variation in the data (adjusted for degrees of freedom). The range in ^{15}N concentration of added N, forms of added N and harvest dates in this experiment provided a check on the effect of these factors on the sample variance. When an analysis of variance was performed on the logarithms of the sample variances it was found that there was no significant difference due to any factor except the ^{15}N concentration of the added N. Therefore rather than calculating weightings for each regression equation (see equation 72), a pooled estimate of the variance for each concentration of ^{15}N in the added N was used. The values for the variances were 3.70×10^{-6}, 4.94×10^{-4}, 7.65×10^{-4} and 1.85×10^{-3} for each increasing ^{15}N concentration of added N, these being about 0.37, 19, 39 and 57 atoms % ^{15}N respectively [see section 10.3.2 for the actual values for $NaNO_3$ and $(NH_4)_2SO_4$].

Table 10.6. Effect of method of calculation on the estimate of the amount of clover nitrogen fixed when grown in association with annual ryegrass. The number of observations used to calculate each value in pot experiment 3 was 12, 24 and 36, and in pot experiment 4 was 4, 12 and 16, for natural abundance, isotope dilution and a_n methods, respectively.

	Shoots only[1]		Whole plants	
	mgN fixed pot^{-1}	(S.E.)	mgN fixed pot^{-1}	(S.E.)
a) POT EXPERIMENT 3 (days 0-21)				
natural abundance[2]	13.13	(0.58)[3]	13.55	(1.28)
^{15}N isotope dilution[2]:				
+0.46 mgN pot^{-1}	12.75	(0.47)	12.99	(1.08)
+1.54 mgN pot^{-1}	12.49	(0.48)	12.87	(1.09)
a_n[4]:				
+0.46 mgN pot^{-1}	12.76	(0.46)	13.29	(1.13)
+1.54 mgN pot^{-1}	12.85	(0.46)	13.60	(1.08)
b) POT EXPERIMENT 4 (days 0-27)				
natural abundance			33.77	(2.18)
^{15}N isotope dilution:				
+^{15}N enriched $NaNO_3$			34.24	(1.81)
+^{15}N enriched $(NH_4)_2SO_4$			40.65	(2.12)
a_n:				
+^{15}N enriched $NaNO_3$			29.85	(2.19)
+^{15}N enriched $(NH_4)_2SO_4$			28.77	(2.47)

[1] Estimates based on no N present at day 0.

[2] mgN fixed = $\underline{P}$ x N yield, using yield-dependent (whole plants) or conventional (shoots only) estimates of $\underline{P}$ (equations 34 and 32, respectively).

[3] To estimate S.E.D., see footnote Table 10.4.

[4] Estimates based on equation 61 (section 10.2).

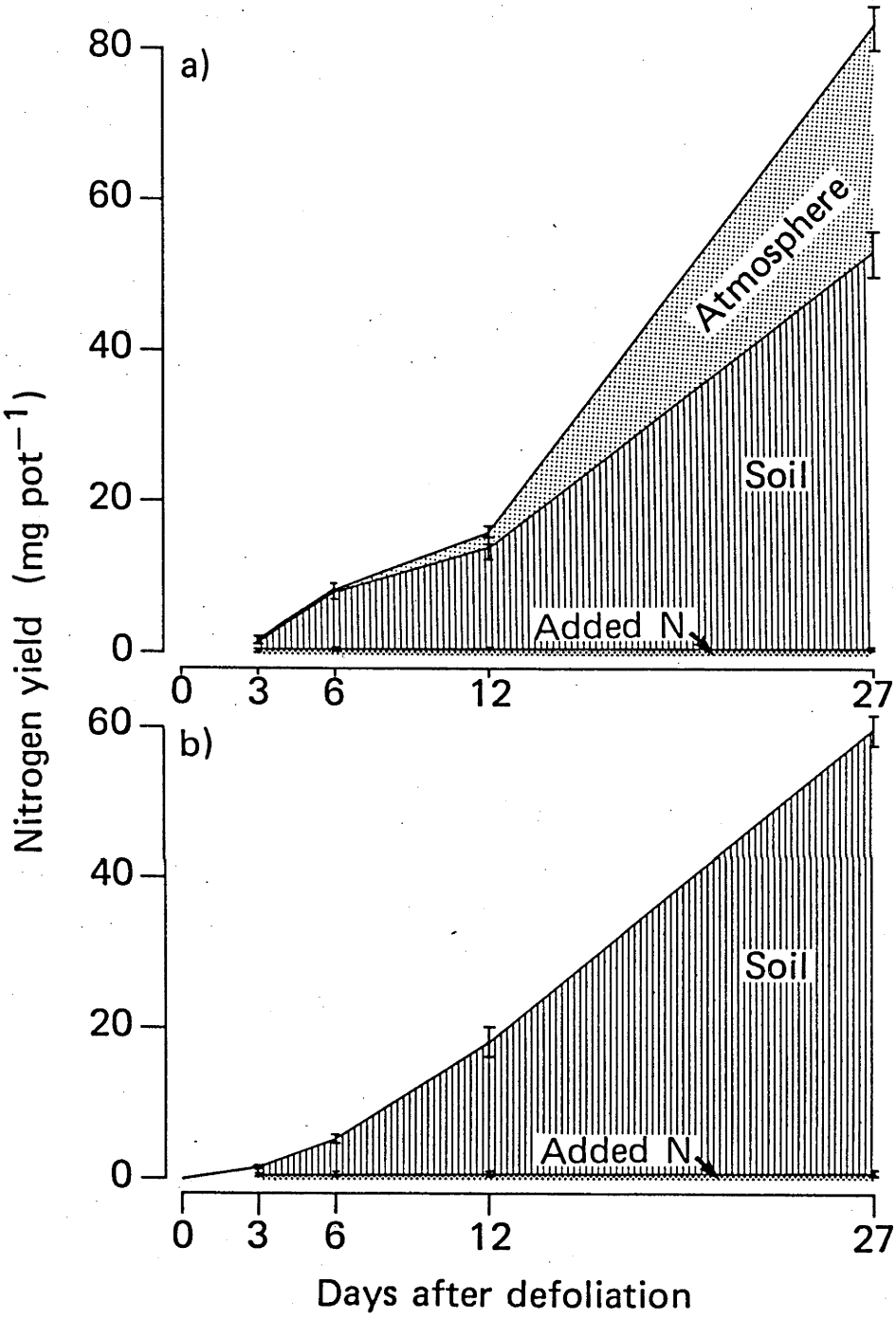


Figure 10.2. Changes with time in the contribution of the various sources of nitrogen for a) subterranean clover and b) annual ryegrass (pot experiment 4). Values were calculated, as described in section 10.2. Each value was calculated from 16 observations. Bars represent ± 1 S.E.

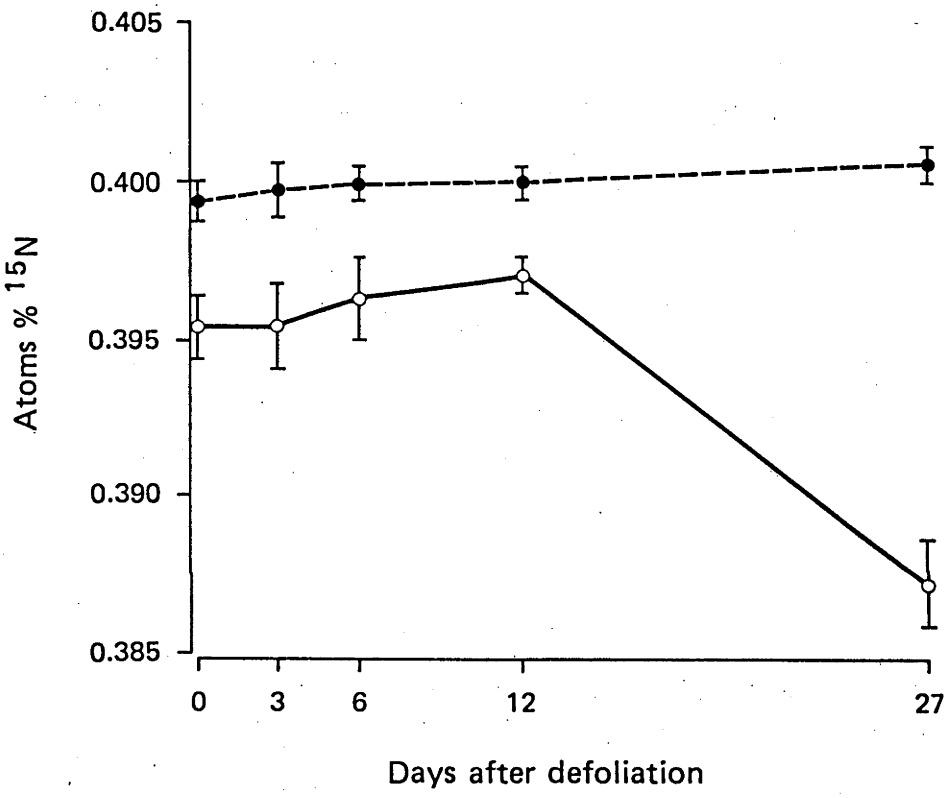


Figure 10.3. Changes with time in the isotopic composition of subterranean clover (○) and annual ryegrass (●) in pots receiving no added nitrogen (pot experiment 4). Each value is the mean of four replicates. Bars indicate ± 1 S.E.

When $NaNO_3$ was applied, 1.5% of the N taken up by the grass by days 3 and 6 was derived from the added N and this decreased to 1.0% by day 27 (Fig. 10.2b). The corresponding values for grass N when $(NH_4)_2SO_4$ was applied were lower, being 1.4% by day 6 and 0.7% by day 27. Prior to day 12, none of the clover N was obtained from the atmosphere, while by day 27 about 40% of the clover N was fixed (Fig. 10.2a). This onset of N_2 fixation by clover during the 12-27 day period is also shown by the decreasing atoms % ^{15}N of clover N in the control treatment (Fig. 10.3). A high plant-available N status of the soil may be the cause of the suppressed N_2 fixation by clover compared with that in pot experiment 3. This is supported by the much lower β (Table 10.5) and $\underline{R}$ values (compare Tables 10.7 and 10.3) in experiment 4 than pot experiment 3.

Clover and grass assimilated the same proportions of added N and soil N during the 27 days after addition of $NaNO_3$ (Table 10.7). However, when $(NH_4)_2SO_4$ was applied the $\underline{R}$ value for clover was lower ($P < 0.05$) than that for the grass at day 27. There was no significant relationship between the individual $\underline{R}$ values and the rates of N assimilation by either clover or grass plants. The errors associated with the measurement of $\underline{R}$ for both clover and grass decreased considerably between days 3 and 27 (Table 10.7). Also, the errors associated with $\underline{R}$ at days 12 and 27 in this experiment were lower than those for the 21 day harvest in pot experiment 3, particularly for clover ($\underline{R}$ experiment 3 $\simeq$ 60 x $\underline{R}$ experiment 4). The latter was largely due to the greater difference in isotopic composition between atmospheric N_2 and soil N in pot experiment 4.

The difference in $\underline{R}$ between clover and grass when the soil N is labelled with $(NH_4)_2SO_4$ but not with $NaNO_3$, is supported by the

Table 10.7. Ratio of added nitrogen to soil nitrogen (R) assimilated by subterranean clover and annual ryegrass as influenced by form of added nitrogen and harvest time (days after nitrogen addition) (pot experiment 4). Each value was calculated using 16 observations.

Form of added N	Harvest time	Grass R	Clover R	Grass R - Clover R	S.E.D.
$NaNO_3$	3	0.0153 (0.0027)[1]	0.0181 (0.0081)	-0.00280	0.00853
	6	0.01523 (0.00065)	0.01277 (0.00167)	0.00246	0.00179
	12	0.01312 (0.00035)	0.01321 (0.00074)	-0.00009	0.00082
	27	0.01059 (0.00026)	0.00972 (0.00059)	0.00087	0.00065
$(NH_4)_2SO_4$	6	0.01388 (0.00055)	0.01021 (0.00147)	0.00367	0.00157
	27	0.00747 (0.00027)	0.00575 (0.00051)	0.00172	0.00058

[1] See footnote, Table 10.3.

values calculated from the same data for the proportion of N fixed (Table 10.5). The P value calculated by ^{15}N isotope dilution, was similar for treatments with added $NaNO_3$ and for natural ^{15}N abundance where there was no N addition and therefore no requirement concerning R. However, similar calculations for $(NH_4)_2SO_4$ resulted in a significantly ($P < 0.05$) higher value of P. Similarly, there was no significant difference between treatments in the estimates of the amount of clover N fixed from atmospheric N_2 using natural ^{15}N abundance, ^{15}N isotope dilution or the method proposed in section 10.2, except for a larger estimate with the ^{15}N-enriched $(NH_4)_2SO_4$ treatment using ^{15}N isotope dilution (Table 10.6).

10.4.3 Soil profile experiment

10.4.3.1 Main study (estimation of R)

10.4.3.1.1 Plant nitrogen accumulation and isotopic composition

Application of the equivalent of 1 kg N ha^{-1} had no significant effect on the amount of N assimilated by clover or its associated grass, as was found in all the other experiments (see Chapter 7). The amount of N removed when the shoots were trimmed (day 0) was 133 and 80 mg N pot^{-1} for ryegrass and phalaris respectively. A similar relative difference between ryegrass and phalaris remained in the stubble N after trimming and was maintained throughout the measurement period (Fig. 10.4a). Conversely, the initial N yield of clover grown with phalaris was greater than when grown with ryegrass and this difference remained throughout the growth period (Fig. 10.4a). However, during the 4-8 day period the rate of N assimilation by ryegrass was greater than that by phalaris and the N assimilation rate for clover grown with ryegrass was less than when grown with phalaris (Fig. 10.4b).

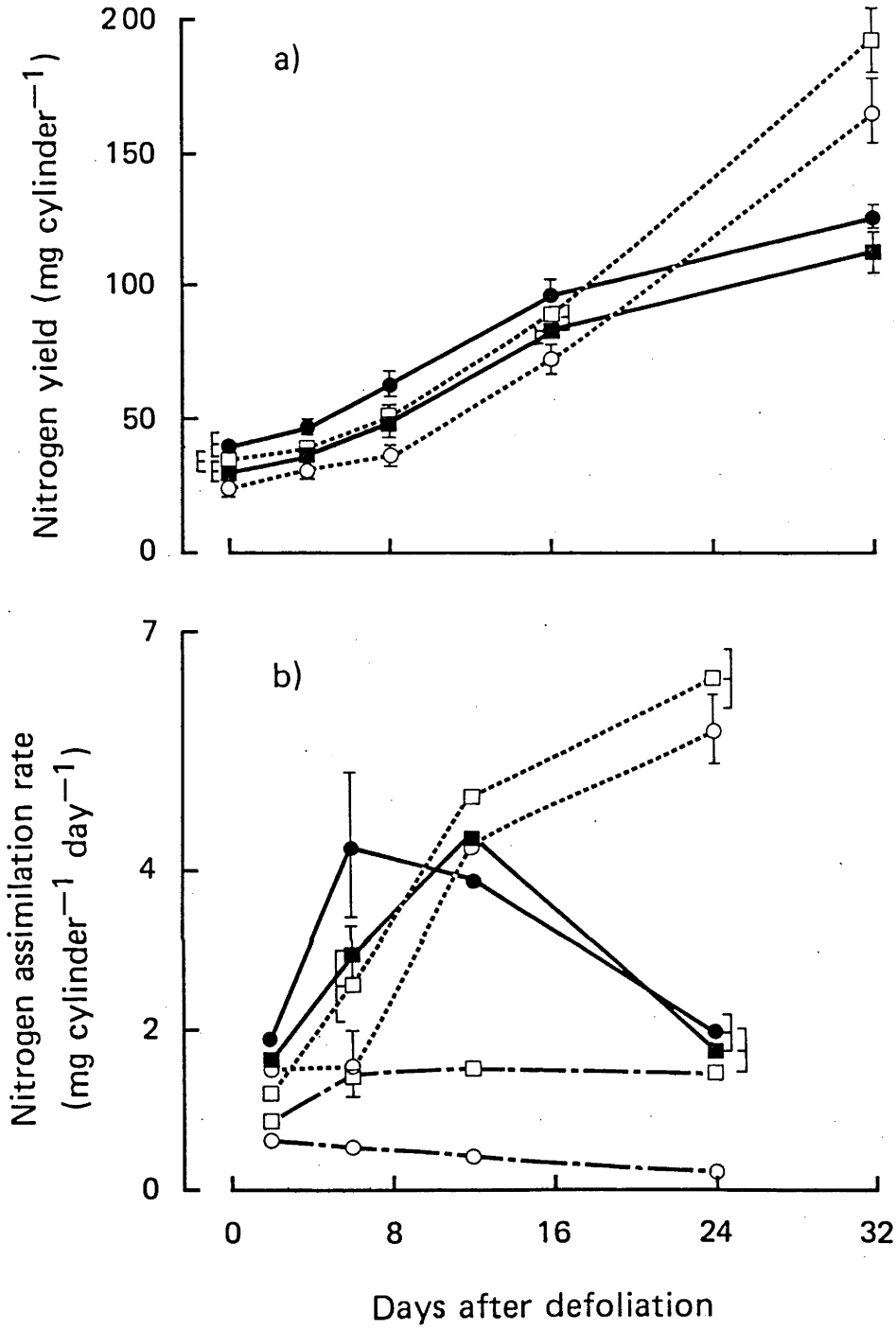


Figure 10.4. Changes with time in a) the amount of nitrogen and b) the rate of nitrogen assimilation by subterranean clover (○) grown with annual ryegrass (●) and subterranean clover (□) grown with phalaris (■) (soil profile experiment). Values for clover refer to total nitrogen (- - - -) or soil-derived nitrogen (——·). Each value is the mean of 16 replicates. Bars indicate ± 1 S.E.

Where no ^{15}N-labelled N was applied, both grasses contained about 0.375 atoms % ^{15}N and this remained relatively constant during the 32 day period (Fig. 10.5a). The natural abundance of ^{15}N in clover grown with ryegrass was significantly lower than when grown with phalaris at all harvests except day 4. In both cases the relative difference between clover and grass increased with time after defoliation (Fig. 10.5a).

10.4.3.1.2 Plant uptake of added and indigenous soil nitrogen

When ^{15}N labelled N was added to the soil, there was a highly significant ($P < 0.001$) relationship between the isotopic composition of added N and plant N. This is confirmed by the small errors associated with the estimates of the slopes (β) of the regression lines (Table 10.8). By day 4 after N addition, 8% of the ryegrass N was derived from the added N and this decreased to 2% by day 32 (Fig. 10.6b). With phalaris, the corresponding values were 4 and 1% respectively (Fig. 10.7b). The greater uptake of added N by ryegrass is also shown by the β and $\underline{R}$ values (Tables 10.8 and 10.9, respectively); the values for ryegrass were 2-3 times higher than those for phalaris. Similarly, the β and $\underline{R}$ values for clover grown with ryegrass generally exceeded those for clover grown with phalaris (Tables 10.8 and 10.9). These effects are probably associated with the lower levels of inorganic soil N available for plant uptake in the clover/ryegrass profiles (Table 10.10), which lead to a higher ^{15}N enrichment of the available N pool.

There was no significant difference between the estimates of $\underline{R}$ for clover and ryegrass when grown together, at any harvest date (Table 10.9). However, the $\underline{R}$ values for clover were significantly higher than

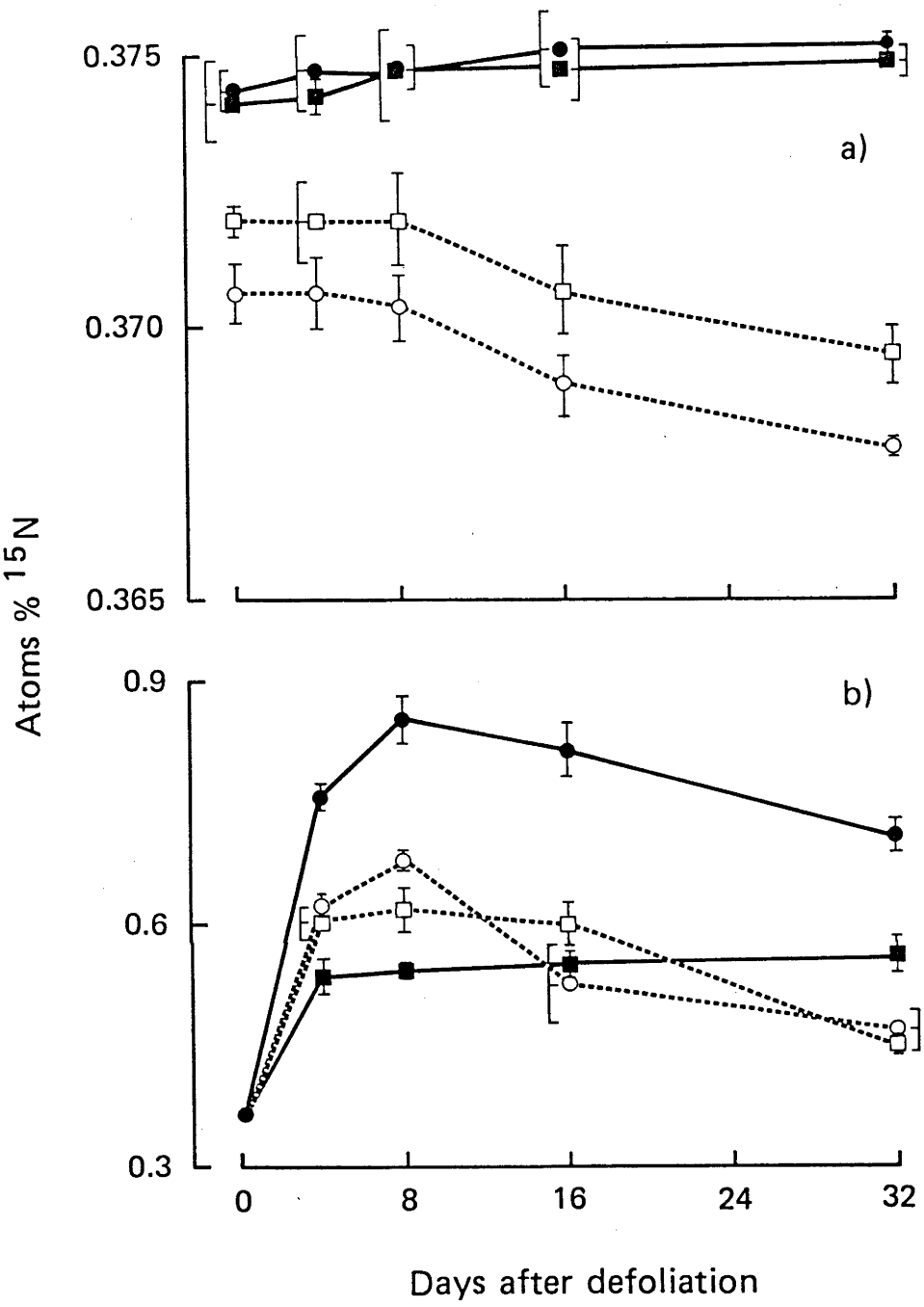


Figure 10.5. The isotopic composition of plant nitrogen in a subterranean clover (○)/annual ryegrass (●) association and a subterranean clover (□)/phalaris (■) association in cylinders receiving a) no added nitrogen or b) KNO_3 (1 kg N ha^{-1}, 23 atoms % ^{15}N) (soil profile experiment). Each value is the mean of four replicates. Bars indicate ± 1 S.E.

Table 10.8. The intercepts (α) and slopes (β) of the regression lines relating the isotopic composition of plant nitrogen to that of added nitrogen when subterranean clover (C) was grown in association with either phalaris (P) or annual ryegrass (R) (soil profile experiment). Each value was calculated using 16 observations.

	Clover		Grass	
	α	$\beta(\times 10^{-3})$	α	$\beta(\times 10^{-3})$
Day 4:				
C,P	0.3679 (0.0012)[1]	10.75 (0.32)	0.3715 (0.0016)	7.83 (0.43)
C,R	0.3637 (0.0011)	11.48 (0.28)	0.3711 (0.0010)	13.99 (0.39)
Day 8:				
C,P	0.3684 (0.0011)	12.35 (0.39)	0.3742 (0.0008)	7.37 (0.15)
C,R	0.3673 (0.0009)	13.61 (0.24)	0.3660 (0.0005)	21.66 (0.22)
Day 16:				
C,P	0.3674 (0.0005)	9.91 (0.50)	0.3725 (0.0004)	8.54 (0.32)
C,R	0.3672 (0.0011)	7.46 (0.69)	0.3709 (0.0005)	18.72 (0.52)
Day 32:				
C,P	0.3689 (0.0002)	3.49 (0.19)	0.3730 (0.0010)	8.14 (0.44)
C,R	0.3665 (0.0005)	5.14 (0.29)	0.3700 (0.0007)	14.76 (0.60)

[1]See footnote, Table 10.4.

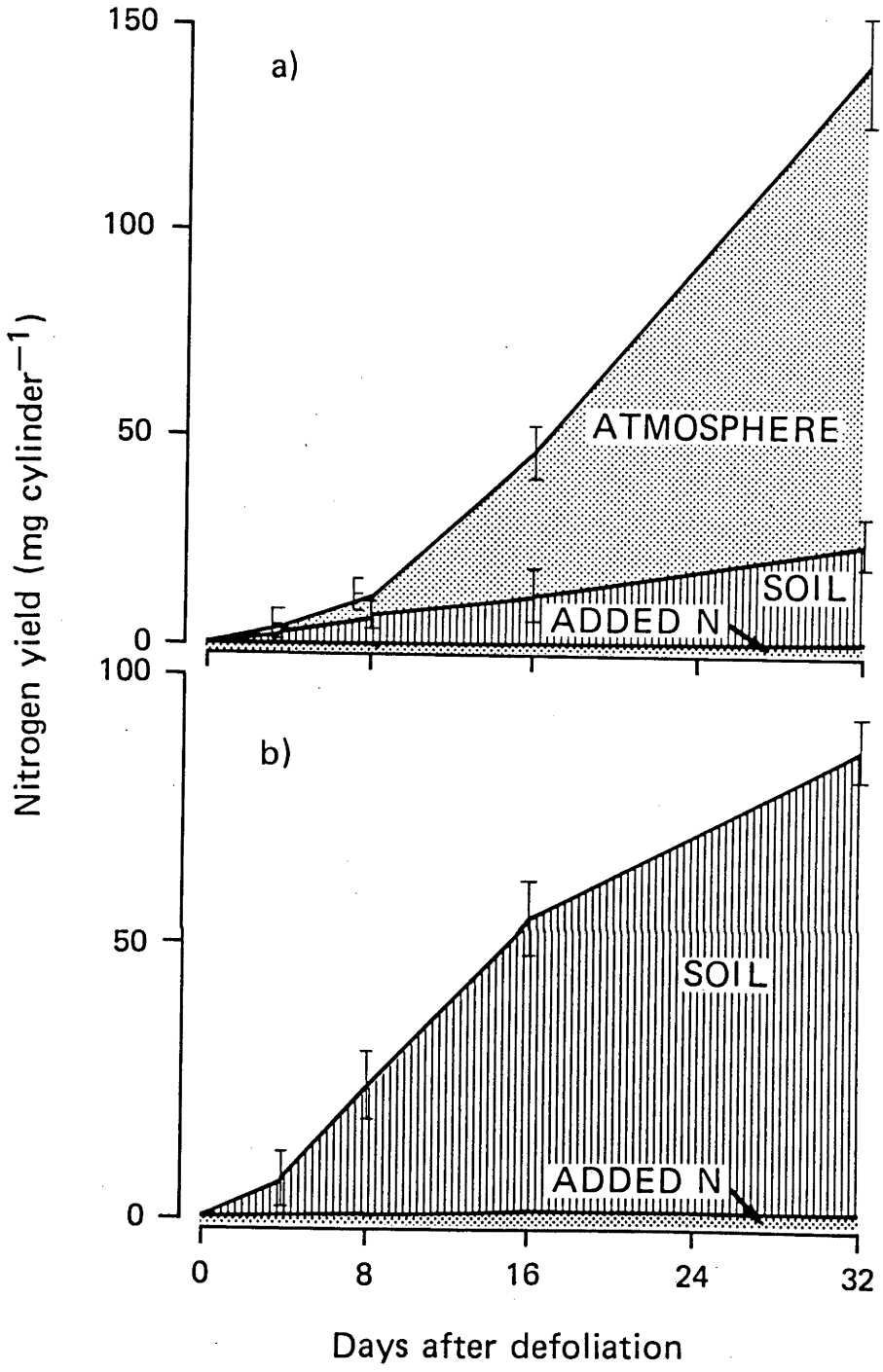


Figure 10.6. Changes with time in the contribution of the various sources of nitrogen for a) subterranean clover and b) annual ryegrass (soil profile experiment). Values were calculated, as described in section 10.2. Each value was calculated from 16 observations. Bars indicate ± 1 S.E.

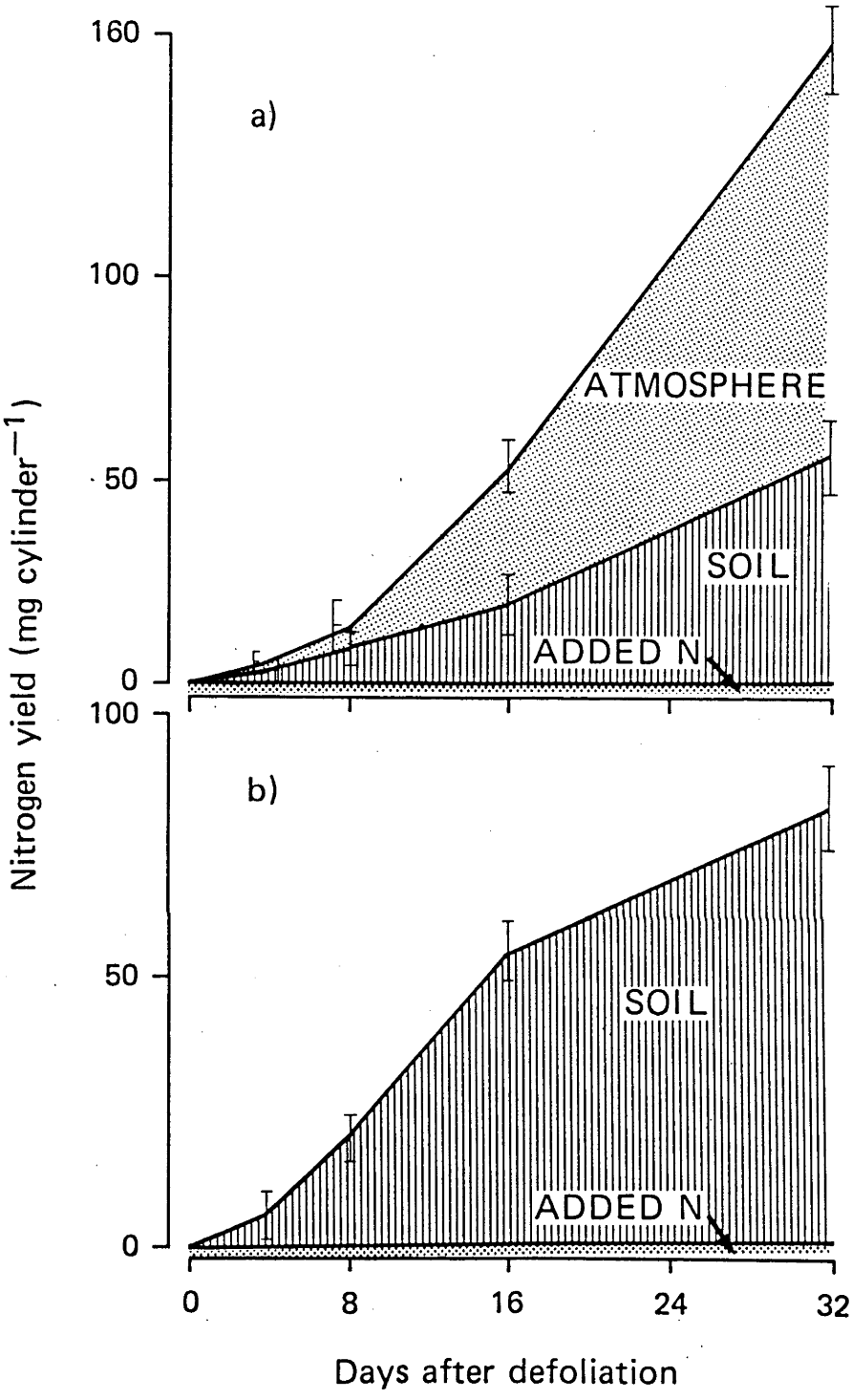


Figure 10.7. Changes with time in the contribution of the various sources of nitrogen for a) subterranean clover and b) phalaris (soil profile experiment). Values were calculated, as described in section 10.2. Each value was calculated using 16 observations. Bars indicate ± 1 S.E.

Table 10.9. Ratio of added nitrogen to soil nitrogen ($\underline{R}$) assimilated by plants when subterranean clover (C) was grown with either phalaris (P) or annual ryegrass (R) in cylinders (soil profile experiment). Each value was calculated using 16 observations.

	$\underline{R}_{grass}$	$\underline{R}_{clover}$	$\underline{R}_{grass}$-$\underline{R}_{clover}$	S.E.D.
Day 4:				
C,P	0.0464 (0.0356)[1]	0.1582 (0.2320)	-0.1118	0.2347
C,R	0.0917 (0.0706)	-0.0661 (0.0328)	0.1578	0.0778
Day 8:				
C,P	0.0186 (0.0041)	0.0630 (0.0166)	-0.0444	0.0170
C,R	0.0591 (0.0153)	0.0658 (0.0419)	0.0067	0.0446
Day 16:				
C,P	0.0133 (0.0012)	0.0445 (0.0109)	-0.0312	0.0110
C,R	0.0322 (0.0033)	0.0452 (0.0308)	-0.0130	0.0310
Day 32:				
C,P	0.0112 (0.0009)	0.0120 (0.0016)	-0.0008	0.0018
C,R	0.0217 (0.0016)	0.0351 (0.0122)	-0.0134	0.0123

[1] See footnote, Table 10.5.

Table 10.10. Inorganic nitrogen and ^{15}N in soil from cylinders growing subterranean clover (C) and either phalaris (P) or annual ryegrass (R), following the addition of KNO_3 (68 atoms % ^{15}N) at 1 kg N ha^{-1} (soil profile experiment). Values for inorganic nitrogen refer to unamended treatments. Each value is a mean of three replicates.

Soil depth (mm)	Days after KNO_3 addition: 4 C,P	4 C,R	8 C,P	8 C,R	16 C,P	16 C,R	32 C,P	32 C,R
Inorganic N (mg g^{-1} soil):								
0-25	4.73	3.54	3.20	2.68	2.65	2.17	2.81	1.17
25-50			2.54	2.55	1.26	0.68	0.83	0.72
50-100	2.86	3.07	3.97	2.94	1.18	0.67	0.54	0.50
100-150	1.79	1.45	2.01	1.46	1.25	0.27	0.36	0.29
150-200	1.53	1.34	1.75	0.80	1.33	0.30	0.37	0.24
200-400	1.68	1.02	1.18	0.49	1.69	0.28	0.25	0.10
S.E.D.	0.49	0.54	0.39	0.43	0.34	0.31	0.38	0.32
Atoms % ^{15}N excess:[1]								
0-25	7.456	9.756	5.529	6.355	5.283	5.325	4.285	2.992
25-50			0.433	0.787	0.642	1.029	1.000	0.731
50-100	0.849	1.352	0.134	0.326	0.255	0.453	0.196	0.096
100-150	0.311	0.324	0.025	-0.028	0.195	0.222	0.154	0.130
150-200	0.027	0.029	-0.024	0.020	0.160	0.098	0.034	0.051
200-400	0.016	0.023	0.006	0.004	0.016	0.039	0.030	0.032
S.E.D.	0.451	0.423	0.410	0.427	0.304	0.326	0.261	0.241

[1] In excess of control.

those for phalaris when they were grown together, at days 8 and 16 but not at day 32. At day 4 there was a large difference in R between clover and phalaris when grown in association, but errors were high due to the small increase in N yield during the 0-4 day period and therefore the difference was not significant (Table 10.9).

10.4.3.1.3 Errors associated with the calculation of R

The errors associated with the values for α and β did not change appreciably with time and were similar for clover and the associated grass (Table 10.8). In contrast, the errors associated with R decreased markedly with increasing growth period (see Tables 10.7 and 10.9). This is due to the corresponding increase in the difference between the initial and final N yields. Within any one time period the errors associated with R were also greater for clover than for the associated grass (Table 10.9). The errors associated with R in this experiment were much greater than those in pot experiment 4 ($\sim$ 6-20 times), particularly with clover (compare Tables 10.7 and 10.9). However, there was little difference between the errors associated with either the α or β values in these two experiments. Errors associated with the change in plant N yield over the 32 day period of the soil profile study were 1.5-2.5 times those obtained during the 27 day period of pot experiment 4. Thus, the greater errors associated with R for clover in the soil profile study were mainly due to the difference in ^{15}N concentration between atmospheric N2 and plant-available soil N being smaller than in pot experiment 4. This is readily seen by comparing the values obtained for α in the two experiments (compare Tables 10.4 and 10.8).

10.4.3.1.4 Estimation of N_2 fixation

The difference between the R values for clover and phalaris, when grown together, at days 8 and 16 is supported by the estimates of P (Table 10.11). Estimates of P using ^{15}N isotope dilution were small and often negative in contrast to those for natural ^{15}N abundance for the clover/phalaris association at days 4,8 and 16. This was associated with the ^{15}N concentration of clover being significantly higher than that for phalaris during this period (Fig. 10.5b). However, at day 32 there was no difference between the R values for clover and phalaris or the P values whether calculated by the ^{15}N isotope dilution or natural ^{15}N abundance methods. With the clover/ryegrass association, there was no significant difference between estimates of P obtained from the ^{15}N isotope dilution and natural ^{15}N abundance methods, except at day 4 for the added N treatments at 23 and 68 atoms % ^{15}N and at day 32 for the 68 atoms % ^{15}N treatment (Table 10.11).

All estimates of P using the conventional method of calculation (equations 31 and 32, Chapter 3) for clover grown with ryegrass were significantly higher than those for clover grown with phalaris (Table 10.12). In all cases, there was no significant difference between estimates of P using conventional or yield-dependent methods of calculation (equations 32 and 34 in Chapter 3, respectively), as also occurred in the field experiment (Chapter 9). The errors associated with P derived by yield-dependent and conventional calculations decreased as the measurement period increased (Tables 10.11 and 10.12).

Measurement of the amount of N (±S.E.) in the roots of each soil layer were variable and averaged 56 (26), 11 (7) and 4 (3) mg for clover and 34 (10), 11 (9) and 6 (3) mg for the grasses at the 0-100,

Table 10.11. Estimates of the percentage of nitrogen fixed using conventional (Conv.) and yield-dependent (Y-D) calculations (equations 32 and 34, respectively) for subterranean clover grown in association with either phalaris or annual ryegrass at natural ^{15}N abundance (nat.ab.) or after the addition of KNO_3 (1 kg N ha^{-1}) at 1, 23 or 68 atoms % ^{15}N (soil profile experiment). All values are for plant shoots, except those in brackets which are for shoots + roots, and represent a mean of four replicates.

	Atoms % ^{15}N of added N	Clover, phalaris		Clover, ryegrass	
		Conv.	Y-D	Conv.	Y-D
Day 0-4:					
nat.ab.		29.9	31.4	49.8	60.1
+ ^{15}N	1	4.0	-37.8	51.9	58.9
	23	-47.1	-100.7	24.9	37.0
	68	-15.4	-64.9	4.4	20.0
S.E.D.		17.2	46.3	8.9	40.7
Days 0-8:					
nat.ab.		34.4	39.9	52.7	61.9
+ ^{15}N	1	6.3	-20.1	40.5	27.6
	23	-42.1	-89.4	36.4	25.0
	68	-94.9	-155.1	47.9	38.8
S.E.D.		14.5	24.7	6.9	19.4
Days 0-16:					
nat.ab.		50.0(45.1)	60.9 (51.3)	71.7(65.0)	80.8(72.6)
+ ^{15}N	1	19.6	23.4	64.6	68.6
	23	-6.5(10.5)	-11.9(-7.6)	63.5(71.1)	67.0(77.5)
	68	-25.9(-15.3)	-32.2(-20.1)	64.1(65.4)	67.8(66.6)
S.E.D.		11.2	18.8	7.5	9.6
Days 0-32:					
nat.ab.		64.4	71.7	85.5	91.0
+ ^{15}N	1	59.9	65.2	75.0	79.4
	23	59.2	63.6	69.8	75.3
	68	53.0	58.1	63.1	69.8
S.E.D.		9.4	13.4	6.6	8.3

Table 10.12. Estimates of the percentage of nitrogen fixed using conventional and yield-dependent calculations (equations 32 and 34, respectively) for subterranean clover (C) as influenced by its association with either phalaris (P) or annual ryegrass (R) at natural ^{15}N abundance (nat.ab.) or after ^{15}N addition (soil profile experiment). Each value is a mean of four and twelve observations for nat.ab. and + ^{15}N, respectively.

	Conventional			Yield-dependent		
	C,P	C,R	S.E.D.	C,P	C,R	S.E.D.
Day 0:						
nat.ab.	29.4	47.2	5.1			
Day 0-4:						
nat.ab.	29.9	49.8	9.4	31.4	60.1	36.2
+ ^{15}N	-19.5	27.1	15.6	-67.8	38.6	47.3
Day 0-8:						
nat.ab.	34.4	52.7	6.8	39.9	61.9	13.4
+ ^{15}N	-43.6	41.6	13.9	-88.2	30.5	28.1
Day 0-16:						
nat.ab.	50.0	71.7	8.6	60.9	80.8	10.4
+ ^{15}N	-4.3	64.1	10.1	-6.9	67.8	16.9
Day 0-32:						
nat.ab.	64.4	85.5	7.1	71.7	91.0	10.3
+ ^{15}N	57.4	69.3	5.8	62.3	74.8	8.5

100-200 and 200-400 mm soil depths. Analysis of plant roots for ^{15}N concentration showed similar trends to those in the field experiment (Chapter 9) with the natural abundance of ^{15}N in the roots of clover and grasses being higher than for shoots whereas this difference between roots and shoots was reversed when the soil was labelled with $^{15}NO_3^-$. Thus, as in the field experiment, there was no significant difference in the estimates of P obtained using data for whole plants (shoots + roots) or shoots-alone (Table 10.11).

The yield-dependent estimates of P using natural ^{15}N abundance data during the 4-8, 8-16 and 16-32 day periods were 41.4, 69.2 and 77.4% for the clover/phalaris association, and 67.3, 90.5 and 96.2% for the clover/ryegrass association respectively. Estimates of the rates of assimilation of N from the soil by clover were derived from these data and are given in Fig. 10.4b. Estimates of the amounts of N_2 fixed by clover were similar when the natural ^{15}N abundance and new (see section 10.2) methods were used but lower when the ^{15}N isotope dilution method was used on data for the clover/phalaris association at days 8 and 16 (Table 10.13).

10.4.3.1.5 ^{15}N concentration of plant-available soil nitrogen

Regular measurement of the ^{15}N concentration of inorganic soil N with depth (Table 10.10) showed that the added $^{15}NO_3^-$ was largely confined to the 0-50 mm soil depth and that most of it remained in the 0-25 mm depth. The ^{15}N concentration of the plant-available soil N also decreased with time after addition of $K^{15}NO_3$ (Fig. 10.8 and Table 10.10, 0-25 mm depth). This decrease in ^{15}N concentration was calculated in an exponential form as

Table 10.13. Effect of method of calculation on the estimate of the amount of clover nitrogen fixed from atmospheric N_2 when grown in association with either phalaris or annual ryegrass (soil profile experiment). Each value was calculated using 4, 12 and 16 observations for natural abundance, isotope dilution and a_n methods, respectively.

	Clover, phalaris	Clover, ryegrass
	mg N fixed cylinder^{-1} (S.E.)	mg N fixed cylinder^{-1} (S.E.)
Days 0-8:		
natural abundance[1]	3.95 (3.76)[3]	4.01 (4.33)
^{15}N isotope dilution[1]	-13.28 (6.99)	3.77 (4.76)
a_n[2]	4.80 (6.24)	4.06 (4.74)
Day 0-16:		
natural abundance	30.41 (8.37)	38.32 (8.39)
^{15}N isotope dilution	- 3.45 (9.00)	32.15 (6.23)
a_n	33.06 (5.20)	34.94 (8.04)
Days 0-32:		
natural abundance	113.6 (13.5)	128.2 (12.8)
^{15}N isotope dilution	98.7 (10.2)	105.4 (12.4)
a_n	101.9 (9.0)	115.7 (13.0)

[1] mg N fixed = $\underline{P}$ x N yield, using yield-dependent $\underline{P}$ values (equation 34, Chapter 3).

[2] Estimated using equation 61 (section 10.2).

[3] To estimate S.E.D., see footnote Table 10.4.

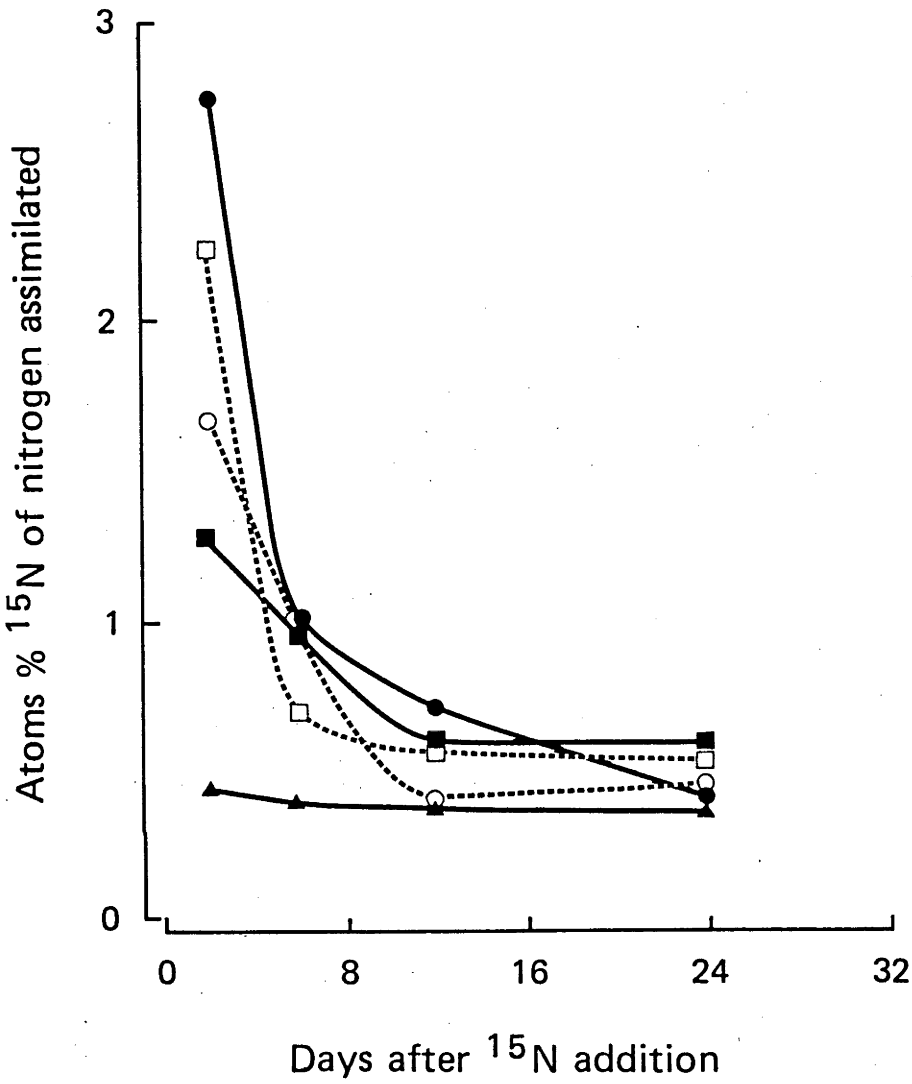


Figure 10.8. Isotopic composition of nitrogen assimilated by subterranean clover (○) grown with annual ryegrass (●) and subterranean clover (□) grown with phalaris (■) after the addition of KNO_3 (1 kg N ha^{-1}, 23 atoms % ^{15}N) at day 0. Data are also given for annual ryegrass (▲) that received KNO_3 at 1 atoms % ^{15}N at day 0. Values were calculated using equation 33 (Chapter 3) and are means of four replicates.

$$\text{atoms \% } {}^{15}N_{soil} = m + ne^{-Dt} \quad (73)$$

where m = atoms % ^{15}N at time infinity (taken as natural abundance), n = atoms % ^{15}N excess at time zero, t = time in days and D = decline constant (Witty 1983b). In order to fit measured data to this expression it was rearranged to give

$$Y = \log_e (\text{atoms \% } {}^{15}N_{soil} - m) = \log_e n - Dt + \text{error}. \quad (74)$$

Using regression analyses, values obtained for D (±S.E.) were 0.025 (±0.003, r^2 = 0.77) and 0.015 (±0.003, r^2 = 0.56) for data on the ^{15}N concentration of inorganic soil N in the 0-25 mm layer for clover/ryegrass and clover/phalaris respectively. This significant difference indicates that the decrease in ^{15}N concentration of available N with time was greatest under the clover/ryegrass association. This is supported by the corresponding values for D using data on the ^{15}N concentration of N assimilated by ryegrass and phalaris (Fig. 10.8), being 0.026 (±0.003, r^2 = 0.92) and 0.018 (±0.005, r^2 = 0.45) respectively.

10.4.3.2 Root activity study

The natural abundance of ^{15}N in ryegrass N was the same as that in phalaris N on days 4 and 16 after trimming, whereas the ^{15}N concentration was lower in clover grown with ryegrass than in clover grown with phalaris at both harvests; the same effect also occurred in the associated main study on the estimation of R (compare Table 10.14 and Fig. 5a). Injection of ^{15}N-labelled NO_3^- into the soil at 50, 150 and 300 mm depths caused a significant ($P < 0.01$) increase in the ^{15}N concentration of N in both grasses and generally increased that in clover. In the clover/phalaris association there was no significant

Table 10.14. Effect of injection of ^{15}N-labelled KNO_3 at 50, 150 and 300 mm soil depths on the ^{15}N concentration of nitrogen in the shoots of clover, phalaris and annual ryegrass, and on the estimate of the proportion (P) of clover nitrogen fixed 4 and 16 days after injection (soil profile experiment). Each value is the mean of six replicates.

	Clover, phalaris			Clover, ryegrass		
	Atoms % ^{15}N			Atoms % ^{15}N		
	Clover	Phalaris	P(%)	Clover	Ryegrass	P(%)
Day 4:						
$+^{15}N$, 50 mm	0.3844	0.4064	55.1	0.3875	0.4255	64.4
$+^{15}N$, 150 mm	0.3838	0.4006	49.4	0.3733	0.4709	93.5
$+^{15}N$, 300 mm	0.3824	0.4025	55.9	0.3710	0.4617	95.3
no added N	0.3676	0.3685	47.1	0.3673	0.3686	64.0
S.E.D.	0.0041	0.0081	9.7	0.0051	0.0185	7.1
Day 16:						
$+^{15}N$, 50 mm	0.4091	0.4436	44.8	0.4061	0.4469	50.8
$+^{15}N$, 150 mm	0.4194	0.4125	-15.0	0.3934	0.4630	72.1
$+^{15}N$, 300 mm	0.3968	0.4311	53.0	0.3782	0.4502	86.1
no added N	0.3674	0.3678	34.4	0.3669	0.3679	71.9
S.E.D.	0.0086	0.0118	9.1	0.0057	0.0194	8.3

effect of depth of ^{15}N injection on the ^{15}N concentrations of either clover or phalaris N on day 4 (Table 10.14). The ^{15}N concentration in clover N from the clover/ryegrass association was higher when ^{15}N was injected at 50 mm than at 150 or 300 mm depths. Conversely, the ^{15}N concentration of ryegrass was lower when $^{15}NO_3^-$ was injected at the 50 mm depth than at 150 or 300 mm (Table 10.14).

By day 16, there was no significant difference between the ^{15}N concentration of ryegrass N at any injection depth. However, the ^{15}N concentration of clover N, when grown with ryegrass, still decreased with increasing depth of ^{15}N injection (Table 10.14).

When $^{15}NO_3^-$ was injected into the soil at 50 mm, estimates of $\underline{P}$ from the day 4 data for the clover/ryegrass association were similar to those obtained from the natural abundance data (Table 10.14). However, when $^{15}NO_3^-$ was injected at 150 and 300 mm the estimates of $\underline{P}$ were much higher. The depth of injection had no effect on the estimates obtained for $\underline{P}$ on day 4 when clover was grown with phalaris. By day 16, estimates of $\underline{P}$ for clover grown with ryegrass were significantly lower when ^{15}N was injected at 50 mm than for all other treatments. In the clover/phalaris association on day 16, estimates of $\underline{P}$ were again similar in all treatments except for a low value when ^{15}N was injected at 150 mm.

The $\underline{P}$ values obtained from the day 4 data were higher than those obtained from corresponding data for the study on the estimation of $\underline{R}$ (compare Tables 10.12 and 10.14). This may be related to differences between the N status of the 0-100 mm layers of the soils in the two studies (see section 10.3.3.1). However, on day 16, estimates of $\underline{P}$ using natural ^{15}N abundance were similar for the two studies.

Most of the inorganic soil N occurred in the 0-100 mm depth of the soil profiles (Table 10.15).

10.5 Discussion

10.5.1 An alternative method for estimating the amount of N_2 fixed

During the derivation of the expression for the amount of legume N obtained from the indigenous soil N (equation 62), an alternative expression for the amount of legume N fixed from the atmosphere was obtained (see equation 61). This is similar to that used for the calculation of atmospheric N_2 fixed using natural ^{15}N abundance (Amarger et al. 1979; Bergersen and Turner 1983), in being dependent upon $(A-S_1)$. However, this new expression has the advantage that it can be used to determine the amount of N_2 fixed by the legume when ^{15}N-labelled fertilizer is applied. In other work estimating the amount of legume N derived from the atmosphere and from the indigenous soil N (e.g. Deibert et al. 1979; Vose et al. 1982; Rennie and Kemp 1983), there is an implicit assumption that the legume and reference plant assimilate added N and indigenous soil N in the same proportions. When the method proposed in section 10.2 is used it is not necessary to make this assumption because the reference plant is used to estimate only the isotopic composition of the N derived from the indigenous soil N (see equations 61 and 62).

The estimates of the amount of clover N fixed by the proposed method and by the natural ^{15}N abundance method were similar in pot experiments 3 and 4 and in the soil profile experiment (Tables 10.6 and 10.12).

Table 10.15. Inorganic soil nitrogen in soil profiles growing subterranean clover in association with either annual ryegrass or phalaris (root activity study; soil profile experiment). Each value is the mean of three replicates.

Depth of soil layer (mm)	Clover, ryegrass		Clover, phalaris	
	μg g^{-1}	mg $layer^{-1}$	μg g^{-1}	mg $layer^{-1}$
Day 4:				
0-100	3.47	20.0	4.97	28.3
100-200	0.43	3.2	0.96	7.1
200-400	0.26	4.0	0.64	10.0
S.E.D.	0.37	4.4	0.12	1.4
total		27.2		45.4
Day 16:				
0-100	2.25	12.8	3.94	22.5
100-200	0.75	5.5	1.40	10.3
200-400	0.46	7.1	0.89	13.7
S.E.D.	0.21	2.5	0.30	3.5
total		25.4		46.5

10.5.2 Factors affecting the estimation of R

The data from the three experiments described in this chapter allowed an examination of some of the assumptions of the technique proposed for measuring R for the legume and reference plant. In pot experiment 3 there was only a small difference between the isotopic composition of N in subterranean clover derived from atmospheric N_2 (B, Table 5.1) and that from indigenous soil N and this resulted in a relatively large error in the R values for clover. However, when a soil whose total N was in equilibrium with ^{15}N added three years previously was used (pot experiment 4 and the soil profile experiment), the error in R (associated with the difference in isotopic composition between clover N derived from the soil and the atmosphere) was considerably reduced. Thus, the accuracy in determining R for the legume is greatest when soils have a high concentration of ^{15}N in the indigenous soil N.

The errors associated with the ^{15}N concentration of plant N increased as the ^{15}N concentration of the added N increased and the values were weighted to adjust for this. These errors were linearly related to the atoms % ^{15}N of the added N in excess of that of the soil N, indicating that the error was related to variability in the distribution of the added N in the pots. In contrast, the concentration of the plant-available indigenous soil N should have been constant throughout the pots because the soils were mixed before potting.

Another important factor contributing to the large errors in the R values for clover in pot experiment 3 was that almost all of the clover N was fixed from the atmosphere and therefore the uptake of added N and soil N was very small. This caused low values for β (the slope of the regression line relating the isotopic compositions of added N and clover

N). In pot experiment 4, the accuracy in determining R for the legume was improved by measuring β more accurately (by including more levels of ^{15}N enrichment of added N) and the greater amount of plant-available soil N produced a higher β value for the clover (i.e. a lower proportion of N_2 fixed). This indicates that the estimation of R for the legume tends to be more accurate when about 50% of its N is fixed from the atmosphere than when it exceeds 90%.

The accuracy in estimating R increased considerably with time after ^{15}N addition in pot experiment 4 and in the soil profile experiment. Thus, it would appear that, for measurement periods of less than 14 days, this technique is probably too insensitive to be of value unless there are larger increases in the amount of plant N than obtained here.

The use of data for shoots-only rather than data for whole plants resulted in no significant difference in the R values or in the relative difference in R values between clover and grass (Table 10.4). With the data for plant shoots, the simplified (yield-independent) equations for estimating R (see equations 69 and 70, section 10.2) were used and these do not require initial measurement of the N present in the stubble + roots. Therefore fewer parameters need to be measured. Similar considerations apply when determining P with the ^{15}N isotope dilution method using yield-dependent techniques (compare equations 32 and 34, Chapter 3). Thus the method chosen for measurement of R and P will depend on the precision required.

10.5.3 Influence of R on the estimation of P

As the difference between the R values for the legume and reference plant increases, the difference between the actual P value and

that estimated by ^{15}N isotope dilution also increases and this effect is greatest at low P values (Fig. 10.9). Thus, in pot experiment 3, where P was about 90%, any differences in R between clover and ryegrass would have had little effect on the estimate of P.

In pot experiment 4 there was a small but significant difference in R between clover and grass when ^{15}N-labelled $(NH_4)_2SO_4$ was used. This led to an overestimate in the value for P compared with values obtained with $Na^{15}NO_3$ and by natural abundance (51 vs. 42 and 41%, respectively). This could be due to several different causes including 1) differences between the ability of the two plants to assimilate NH_4^+, and 2) differences in ability of clover and grass to assimilate N from different soil depths. Differences between plants in their ability to assimilate NH_4^+ from soil have been documented (e.g. Haynes and Goh 1978). This could also be influenced by the movement of added N in the soil. Movement of NH_4^+ from the site of addition is usually small in most soils, whereas NO_3^- is readily leached (Harmsen and van Schreven 1955; Nommik and Vahtras 1982). Whatever the reason, it would appear that, if ^{15}N-labelled N is to be applied to the soil surface, it is preferable to use NO_3^- rather than NH_4^+ compounds when using the ^{15}N isotope dilution method for measuring N_2 fixation.

In pot experiments 3 and 4 there was a relatively small amount of soil in each pot (1.6-1.9 kg) and within a short time period the roots of clover and ryegrass were spread throughout the soil. This would tend to minimise differences in root patterns between the two plants compared with those found in the field. Because of this, the soil profile experiment was established, so that any differences between plants in their root growth with soil depth could be better expressed. This study enabled a more accurate assessment of R to be made than

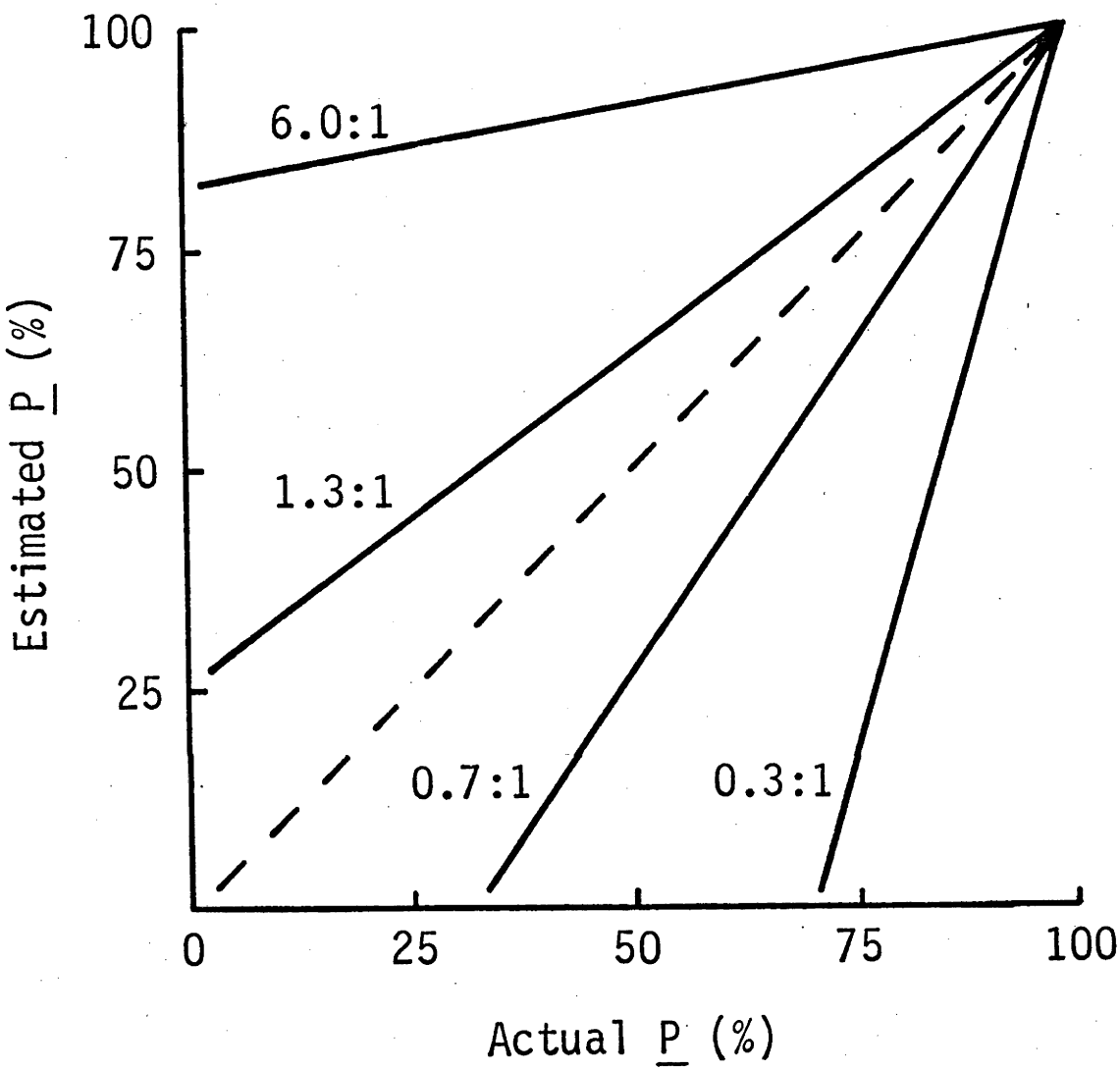


Figure 10.9. Effect of relative differences between reference plant and legume in the ratio of uptake of added nitrogen and indigenous soil nitrogen ($\underline{R}_{\text{reference plant}}$: $\underline{R}_{\text{legume}}$) on the estimates of the proportion ($\underline{P}$) of legume nitrogen fixed. The broken line indicates where estimated $\underline{P}$ = actual $\underline{P}$.

could be done in the field because a constant plant density was used and soil heterogeneity was overcome by using uniformly mixed soil in each profile layer, including one of a relatively high 'natural' ^{15}N enrichment of the soil N.

In the clover/ryegrass association of the soil profile experiment, ryegrass assimilated a greater proportion of the $^{15}NO_3^-$ injected at the 150 and 300 mm depths than at the 50 mm depth, resulting in high P values. Therefore it can be concluded that the ability to absorb N from the 100-200 and 200-400 mm layers was much greater for ryegrass than for clover. However, plant roots were mainly found in the 0-100 mm layer and most of the plant-available soil N was found in this layer (Table 10.15). Thus, any differences in root pattern between plants in the 100-400 mm soil zone would have only had a small effect on the total N uptake and on the estimate of P, and this is supported by the similar R values obtained for clover and ryegrass in the main study when $^{15}NO_3^-$ was applied to the soil surface. A similar effect would have been expected in the field experiment where the amount of plant-available soil N also decreased with soil depth (see section 9.3.5).

The clover/phalaris association in the soil profile experiment produced relatively large differences between the R values obtained for the two species during the first 16 days after ^{15}N addition. This was associated with negative P values being estimated by the ^{15}N isotope dilution method. The negative P values could have been due to:

1) differences between clover and phalaris in their pattern of N uptake with soil depth (Edmeades and Goh 1979; Knowles 1981),
2) differences between the plants in their translocation of ^{15}N from roots to shoots, or

3) differences in their pattern of N assimilation with time in conjunction with changes in the ^{15}N concentration of plant-available soil N (Witty 1983,b).

Measurements of soil inorganic N showed that the added ^{15}N was restricted to the 0-50 mm layer and most of this was in the 0-25 mm layer. However, the associated root activity study revealed that there was no difference between clover and phalaris in the relative uptake of $^{15}NO_3^-$ from 50, 150 and 300 mm soil depths. Also, the natural abundance of ^{15}N in phalaris N was the same as that in ryegrass N, whether the ^{15}N concentration of soil N was similar throughout the 0-400 mm profile or higher in the 0-100 mm layer than in the 100-400 mm zone. This indicates that both grasses obtained similar amounts of N from these two soil layers and yet the differences in R between plants growing together only occurred in the clover/phalaris association. Thus, it would appear that the differences in R between clover and phalaris were not due to differences in their root distribution, although differences in their N uptake from the 0-25 mm zone relative to that from below 25 mm cannot be completely discounted.

Negative estimates of P were obtained when both whole plant and shoots-only data were used and therefore this possibility can be discounted. Similarly, the difference between R values for clover and phalaris and the decrease in this difference with time cannot be attributed to the effect of the initial plant N because yield-dependent estimates of R were used.

It would appear that the negative P value were a result of changes in the ^{15}N enrichment of plant-available soil N with time in conjunction with changes in the pattern of N assimilation by clover and phalaris. The ^{15}N concentration of inorganic soil N (Table 10.10) and N

assimilated by the grasses (Fig. 10.8) decreased with time after ^{15}N addition, the largest decline occurring during the 0-8 day period. This decrease in ^{15}N enrichment was greater under the clover/ryegrass association, presumably because of the greater uptake of ^{15}N, than by the clover/phalaris association. Differences in the rate of decline in ^{15}N concentration of N assimilated from the added N + soil N were also generated in this study by using added N at three different levels of ^{15}N enrichment (1, 23 and 68 atoms % ^{15}N; see Fig. 10.8). In the clover/phalaris association on days 4, 8 and 16, and in the clover/ryegrass association on day 4, the estimates of $\underline{P}$ using the ^{15}N isotope dilution method generally decreased as the ^{15}N concentration of the added N increased (Table 10.11). This coincided with differences between the $\underline{R}$ values measured for clover and the associated grass (Table 10.9).

There appeared to be little difference between the patterns of N assimilation of ryegrass or phalaris, or for clover grown in either association (Fig. 10.4a). Some differences between species in the rate of N assimilation occurred between the 0-4 and 4-8 day periods but the differences were more closely matched in the clover/phalaris association than in the clover/ryegrass association. These results refer to total plant N, and include fixed N_2 in the case of the legumes. The requirement described by Witty (1983a,b) is that the ratio of uptake of N from the soil between the legume and reference plant should be constant with time. From Fig. 10.4b it can be calculated that this ratio for clover relative to grass was 0.32, 0.12, 0.11 and 0.11 for the clover/ryegrass association and 0.54, 0.44, 0.35 and 0.84 for the clover/phalaris association for the 0-4, 4-8, 8-16 and 16-32 day periods, respectively. Although the ratio was constant during the 4-32

day period for the clover/ryegrass association, the initial change was similar to the overall variation in the ratios for the clover/phalaris association. These results illustrate that the suitability of different reference plants for obtaining a correct estimate of P cannot always be predicted from simple comparisons of their N accumulation over time relative to those of the legume or even from a comparison with the amounts of N derived from the soil.

The soil profile study has shown that, if the legume and reference plant are not well matched, then a correct estimate of the ^{15}N concentration of the added N + soil N asssimilated by the legume may be obtained only at certain stages of plant growth. Thus, phalaris was shown to be a poor reference plant for subterranean clover when P was estimated by ^{15}N isotope dilution, at least during relatively short growth periods. This supports the suggestion from the field experiment (Chapter 9) that the value of P for clover grown with phalaris obtained by the ^{15}N isotope dilution method may have been underestimated.

The reference plant can also have a real effect on P when grown in association with the legume (Table 10.12). When clover was grown with ryegrass it fixed a greater proportion of its N than when grown with phalaris. This can be attributed to the greater depletion of available soil N by ryegrass than by phalaris. Ryegrass is a fast-establishing grass whereas phalaris is notoriously slow (Langer 1973) and this difference in rate of establishment resulted in ryegrass extracting much more soil N by the start of the measurement period. The higher levels of inorganic N in the soil under the clover/phalaris association (see Table 10.10) resulted in a longer delay in the onset of high levels of N_2 fixation. The effect of increasing levels of inorganic

N in the soil on delaying nodulation and the onset of N_2 fixation, and on reducing P is well documented (e.g. McAuliffe et al. 1958; Munns 1977; Hoglund and Brock 1978). During the measurement period the P values increased, presumably due to depletion of plant-available soil N (Table 10.10). The reduction in plant-available soil N coincided with a decrease in N assimilation rate by the grasses during the latter half of the growth period; this decrease occurred earlier for ryegrass than for phalaris (Fig. 10.4b).

This study illustrated that in mixed plant associations, the reference plant can have a real and an erroneous effect on the estimate of P for a legume using ^{15}N isotope dilution. Thus, the correct interpretation of results obtained from isotope dilution studies requires supportive measurements, including inorganic soil N concentrations, and the use of several treatments such as those involving 1) ^{15}N injection at different soil depths and 2) one rate of N application at different ^{15}N concentrations so that different patterns of decline in ^{15}N enrichment with time are generated. Values of R can also be calculated from the latter treatments and can assist in evaluating reasons for differences in P generated by different ^{15}N-addition treatments or by using different reference plants.

CHAPTER 11

GENERAL DISCUSSION AND CONCLUSIONS

The increased use of the ^{15}N isotope dilution method for estimating N_2 fixation in recent years has occurred with little regard to evaluation of the potential problems of the method. The results obtained from ^{15}N dilution experiments have been accepted as correct, unless obvious anomalies were found. For example, Patterson and LaRue (1983) obtained negative estimates of P when non-nodulating soybeans were used as reference plants for nodulated soybeans. The limited use of the natural ^{15}N abundance method for estimating N_2 fixation in the field has caused it to be described as 'at best, roughly quantitative' (Hauck and Bremner 1976; Bremner and Hauck 1982). The series of experiments described in this thesis identifies and evaluates some of the features of the ^{15}N isotope dilution and natural ^{15}N abundance methods which can cause errors in the estimation of P, by legumes, with special reference to pastures.

In both the field and soil profile experiments the results showed that the most important factor causing erroneous estimates of P by ^{15}N isotope dilution was the ability of the reference plant to measure accurately the isotopic composition of the N assimilated from the soil by the legume. Interpretation of the results from these studies was complicated because of the real effect of the reference plant on the level of soil N available for uptake by the legume, and therefore on P. Nevertheless large differences in the estimate of P by ^{15}N isotope dilution were obtained with different reference plants, even when

growing together in the same plot. Thus, the reference plant may not always assimilate added N and indigenous soil N in the same ratio (R) as the legume, and an erroneous estimate of P can result.

A new technique was developed whereby R could be determined separately for the legume and reference plant. Studies with this technique confirmed the suggestions from the field experiment that phalaris was a poor reference plant for subterranean clover. Furthermore, negative estimates of P were sometimes obtained when the ^{15}N isotope dilution method was used for the clover/phalaris association. This was considered to be due to a decrease with time in the ^{15}N concentration of the plant-available soil N after $^{15}NO_3^-$ addition, and to preferential N assimilation by the legume at an earlier stage of growth compared with phalaris. As a result the ^{15}N concentration in the legume was temporarily higher than that in phalaris. In the clover/ryegrass association, the rate of decline in ^{15}N enrichment of plant-available soil N was even greater than that in the clover/phalaris association. However, there was no difference in R between clover and ryegrass, indicating that annual ryegrass was probably a good reference plant for subterranean clover. It has been suggested that differences in R between legumes and reference plants could also be due to differences in their root penetration and N uptake pattern with soil depth, particularly since the added ^{15}N often remains in the surface layer of soil (e.g. Knowles 1981). However, root activity studies revealed that, in practice, this may be of little importance in established pastures since most plant-available soil N and most roots occur relatively close to the soil surface.

Witty (1983b) also recognised the problem of a declining ^{15}N enrichment of the plant-available soil N and its effect on the

estimation of P by ^{15}N isotope dilution. He suggested that several reference plants be used to gain some feeling of their suitability. However, in established legume/grass pastures there may be little or no choice in the reference plant. In view of the large errors in the estimate of P using ^{15}N isotope dilution that can occur, one or more of the following procedures should be considered to improve the accuracy of the estimate:

1) the use of ^{15}N compounds with slow-release characteristics, as tested by Witty and Ritz (1984), although their addition may result in problems because of poor penetration into the soil when surface-application is necessary;

2) regular additions of small amounts of ^{15}N-labelled inorganic N during the measurement period (the effect of this procedure on changes in ^{15}N concentration of soil N with time on R warrants detailed evaluation);

3) inclusion of several treatments with the same amount of added N but different ^{15}N concentrations, thereby providing differences in the rate of decline in ^{15}N enrichment of soil N. Estimates of P using these treatments should be the same if the legume and reference plant are well matched in their temporal growth patterns. Also, R values may be calculated from these treatments if greater detail is required; and 4) concurrent use of natural ^{15}N abundance or other methods.

In Chapter 4, the natural abundance of ^{15}N in the soil N from a range of soils was found to be sufficiently different from atmospheric N_2 to enable estimation of P. This difference in isotopic composition was very small compared with that where the soil N was enriched by adding ^{15}N-labelled compounds. However, mass spectrometric measurement of the ^{15}N concentration at natural abundance was more

accurate than at high ^{15}N enrichment and the isotopic composition of N assimilated from unamended soil was much more uniform than that from soil to which ^{15}N-labelled compound had been applied. Thus, estimation of P by the natural ^{15}N abundance method was almost as sensitive as that using ^{15}N isotope dilution. Also, it was not fraught with the problems of the ^{15}N isotope dilution method discussed above. In the soil examined, the ^{15}N concentration in plant-available soil N was found to be uniform with soil depth and varied little with time. Thus, reference plants that were unsuitable with the ^{15}N isotope dilution method proved satisfactory with the natural ^{15}N abundance method. The uniformity in the natural abundance of ^{15}N in plant-available soil N with depth and time requires examination in other soils to see if this is a common characteristic.

Isotopic fractionation can have a much greater effect on the estimates of P by natural abundance than by isotope dilution because of the small natural differences in ^{15}N concentration between atmospheric and soil N. Great care was required to avoid isotopic fractionation during sample preparation for ^{15}N analysis and precise analytical capabilities were required. Isotopic fractionation during plant uptake of soil N was not considered important but it was found to be important to establish and use a factor (B) for isotope fractionation during N_2 fixation. For example, the estimate of P using natural abundance for lucerne grown with ryegrass in the field was 81% when B was used, but only 61% when the isotopic composition of the fixed N was considered to be the same as that of atmospheric N_2. Thus it is recommended that B be established for each legume being studied.

The natural ^{15}N abundance method is probably better suited for studies on N_2 fixation in natural ecosytems than the ^{15}N isotope dilution method. It also has the potential to be extended to other systems, such as aquatic environments. For example, measurements of the δ ^{15}N (±S.E.) of Azolla pinnata R.Br. (consisting of a symbiosis of the aquatic water fern Azolla and the N_2-fixing cyanobacterium, Anabaena azollae) and of Salvinia auriculata Aublet. (a water fern from the same family as Azolla but with no N_2-fixing association) found growing together in a pond were 1.40 (±0.41) and 5.42 (±0.27) ‰. Although a value for B was not established for A. pinnata, the differences are large enough to indicate that most of the N in the A. pinnata was fixed from atmospheric N_2. Thus, the use of the natural ^{15}N abundance method warrants further evaluation in systems other than those with crop and pasture legumes.

The research presented has shown than when the ^{15}N isotope dilution and natural ^{15}N abundance methods are used for the estimation of N_2 fixation in the field, it is important that their potential problems be taken into consideration in the design of the experiments and in the interpretation of the results. Only then will more accurate estimates be obtained by these methods.

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APPENDIX 1

DERIVATION OF AN EQUATION FOR ESTIMATING TRANSFER OF NITROGEN FROM A LEGUME TO AN ASSOCIATED GRASS USING ^{15}N-LABELLING OF THE LEGUME

To estimate transfer of N from a legume to an associated grass by ^{15}N-labelling of the legume it is necessary to have two treatments involving a legume/grass association; 1) a control (natural ^{15}N abundance), and 2) legume plant(s) enriched in ^{15}N by foliar absorption at time t_o. The derivation of the proportion of ^{15}N-labelled legume N transferred to the associated grass between times t_o and t_1 is given below (see Table A1.1 for symbols)

The amount of ^{15}N in a plant can be obtained by multiplying the N yield by the atoms % ^{15}N of the plant, e.g. $N_{lo}L_{\ell o}$ for the ^{15}N-labelled legume at time t_o. The extent of the ^{15}N-labelling of a plant can be estimated from the difference in the amount of ^{15}N in plants between the labelled and control treatments, e.g. $N_{lo}L_{\ell o}-N_{lo}L_{uo}$ or $N_{lo}(L_{\ell o}-L_{uo})$ for the legume at time t_o.

Since the ^{15}N-labelling of the legume is complete at t_o, any N assimilated by the legume during the measurement period, t_1-t_o, should be of the same amount and isotopic composition in the labelled and control treatments. Thus, the estimate of the amount of N transferred must be based on the legume N present at t_o. However, the amount of ^{15}N-labelled N in the legume at t_o can be estimated from that present in the legume and the grass (the transferred

component), at t_1. With this estimation, it is assumed that the original ^{15}N-labelled N remains in the legume or is transferred to the associated grass. Thus,

$$N_{1o}\ (L_{\ell o} - L_{uo}) \simeq N_{11}(L_{\ell 1} - L_{u1}) + {}^{15}N \text{ transferred to grass.} \qquad (75)$$

Any transfer of ^{15}N-labelled legume N to the associated grass between t_o to t_1 would result in an increase in the ^{15}N concentration of the grass. The amount of ^{15}N-labelled legume N transferred to the grass is:

$$\text{Transferred N} = (N_{g1}G_{\ell 1} - N_{go}G_{uo}) - (N_{g1}G_{u1} - N_{go}G_{uo}). \qquad (76)$$

However, the amount and isotopic composition of the grass N at t_o should be identical for the two treatments (Table A1.1). Therefore,

$$\text{Tranferred N} = N_{g1}G_{\ell 1} - N_{g1}G_{u1}. \qquad (77)$$

The proportion of legume N transferred to the associated grass (T_L) can then be estimated from data for t_1 using equations 75 and 77, i.e.

$$T_L = \frac{N_{g1}\ (G_{\ell 1} - G_{u1})}{N_{11}(L_{\ell 1} - L_{u1}) + N_{g1}\ (G_{\ell 1} - G_{u1})}\ . \qquad (78)$$

Table A1.1. List of symbols used to denote the amount and isotopic composition of N in legumes and grasses for a control (natural ^{15}N abundance) treatment and a treatment involving foliar labelling of the legume with ^{15}N.

	Natural abundance		^{15}N-labelled legume	
	g N m^{-2}	atoms % ^{15}N	g N m^{-2}	atoms % ^{15}N
Time t_o:				
Legume	N_{lo}	L_{uo}	N_{lo}	$L_{\ell o}$
Grass	N_{go}	G_{uo}	N_{go}	G_{uo}
Time t_1:				
Legume	N_{l1}	L_{u1}	N_{l1}	$L_{\ell 1}$
Grass	N_{g1}	G_{u1}	N_{g1}	$G_{\ell 1}$

APPENDIX 2

DERIVATION OF THE PROPORTION (P) OF LEGUME NITROGEN FIXED FROM ATMOSPHERIC N_2 BY THE CONVENTIONAL CALCULATION USING A FIXED YIELD-DEPENDENT P VALUE.

Yield-dependent estimates of P (equation 34, Chapter 3) adjust for plant N present at the start of a measurement period. Thus, to examine the effect of initial plant N on the estimate of P by the conventional equations (31 and 32, Chapter 3), a procedure was used whereby the conventional calculations could be made on data for a fixed yield-dependent P value.

For a given atoms % ^{15}N value of the reference plant at the end of a measurement period (t_1), the atoms % ^{15}N of the N assimilated during the measurement period (t_1-t_o) was estimated by the yield-dependent equation

$$\text{atoms \% } {}^{15}N_{(t_1-t_0)} = \frac{N_1 \text{ atoms \% } {}^{15}N_{t_1} - N_0 \text{ atoms \% } {}^{15}N_{t_0}}{N_1 - N_0} \qquad (79)$$

where N_o and N_1 are the N yields at t_o and t_1 respectively. The corresponding value for the legume at a fixed yield-dependent P value was obtained by rearranging equation 32 (Chapter 3) to give

$$\text{atoms \% } {}^{15}N_{\text{legume } (t_1-t_0)} = (1-P).\text{atoms \% } {}^{15}N_{\text{ref}(t_1-t_0)} + P.B \qquad (80)$$

where B is the atoms % ^{15}N of the legume N derived entirely from atmospheric N_2 and the subscript ref indicates the reference plant. The atoms % ^{15}N of the legume that would be measured at t_1 can then be obtained by rearranging the equivalent of equation 79 for the legume as follows:

$$\text{atoms \% } {}^{15}N_{\text{legume}(t_1)} = [(NL_1 - NL_o).\text{atoms \% } {}^{15}N_{\text{legume}(t_1-t_o)} + NL_o.\text{atoms \% } {}^{15}N_{\text{legume}(t_o)}]/NL_1 \quad (81)$$

where NL_o and NL_1 are the N accumulated by the legume at t_o and t_1 respectively. Thus, the conventional method of calculating $\underline{P}$ (equation 32, Chapter 3) could then be applied, since values for the atoms % ^{15}N of the legume (equation 81) and reference plant (a fixed value) at t_1 have been obtained.